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Ecotoxicological Effects of Gold Nanoparticles and Microplastics in *Daphnia magna*

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Orientador: Professora Doutora Lúcia Maria das Candeias Guilhermino

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Ecotoxicological Effects of Gold Nanoparticles and Microplastics in *Daphnia magna*

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Orientador – Professora Doutora Lúcia Maria das Candeias Guilhermino

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Resumo

Os nanomateriais (NMs) têm recebido uma crescente atenção tanto em contextos académicos como industriais. As suas propriedades particulares, consequentes do seu tamanho diminuido, apresentam um enorme potencial para aplicações num largo leque de áreas tais como a eletrónica, biomedicina, produtos de higiene pessoal, entre outras. Isto tem motivado um aumento da produção de NMs, tanto para investigação académica como para aplicações industriais e comerciais. À medida que a sua produção aumenta, aumenta também a probabilidade de exposição a estes materiais. Em simultâneo, a consciencialização em relação à presença de microplásticos (MP) no ambiente tem também aumentado. Os MP resultam da degradação de objetos de plástico de maiores dimensões, após uma exposição prolongada a, por exemplo, radiação ultra-violeta e abrasão física. Enquanto que já existe um conhecimento extensivo sobre o potencial tóxico de plásticos à escala macroscópica, os MP só recentemente têm ganho a atenção das entidades reguladoras. Tanto os NMs como os MP apresentam um potencial tóxico considerável que permanece maioritariamente por explorar. O tamanho nanométrico dos NMs poderá conceder-lhes a abilidade de ultrapassar várias barreiras biológicas, infiltrar e danificar elementos sub-celulares, interagir com vários processos celulares ou causar outros tipos de efeitos detrimentais ainda desconhecidos. Alguns destes fenómenos já foram reportados, principalmente em modelos *in vitro* ou modelos *in vivo* com mamíferos. Também já foi mostrado que os MP podem interferir com organismos vivos. A acumulação de MP ingeridos por vários organismos marinhos já foi reportada, embora os efeitos dessa acumulação permaneçam desconhecidos.

Durante o presente trabalho, o potencial tóxico de nanopartículas de ouro (AuNP) e MP de polietileno foi avaliado no cladócero de água doce *Daphnia magna*. Este organismo foi escolhido porque é um dos organismos modelo mais usados na investigação em ecotoxicologia, uma vez que é considerado representativo do zooplâncton e dos consumidores primários em água doce. Com este fim, um ensaio ecotoxicológico crónico foi conduzido com AuNP, MP e com misturas de ambos os tipos de partículas. Numa primeira fase, as metodologias associadas à manutenção de culturas de *D. magna* foram aprendidas e treinadas. Isto incluiu não só o manuseamento e manutenção dos animais, mas também a manutenção de culturas de *Chlorella vulgaris*, uma vez que estas microalgas foram usadas para alimentar as dáfnias durante o trabalho. A segunda fase consistiu na caracterização das AuNP e MP. Foram determinadas curvas de calibração e foram conduzidos ensaios de degradação das partículas em água ultra-pura e no meio de cultura de *D. magna*, usando espectrofotometria ou espectrofluorometria. Finalmente, o ensaio para avaliar a toxicidade crónica em *D. magna* foi conduzido, usando a reprodução como critério de toxicidade. O ensaio teve uma duração de 21 dias, durante os quais a mortalidade dos animais

e vários parâmetros reprodutivos foram monitorizados diariamente. As hipóteses nulas testadas foram: i) concentrações na ordem das ppm de AuNP não são tóxicas para *D. magna*; ii) concentrações na ordem das ppb não são tóxicas para *D. magna* e iii) a precença de MP na água não influencia a toxicidade das AuNP em *D. magna*. Os resultados obtidos sugerem que tanto as AuNP como os MP individualmente podem induzir toxicidade em *D. magna*, causando mortalidade e reduzindo as capacidades reprodutivas dos indivíduos expostos. Também se observou que as AuNP e MP, quando presentes em misturas, parecem interagir entre elas, intensificando o potencial tóxico observado em *D. magna*. Consequentemente, as três hipóteses nulas testadas foram rejeitadas.

Palavras-chave: *Daphnia magna*,, nanopartículas de ouro, nanotecnologia, microplásticos, toxicidade crónica, toxicidade de misturas,

Abstract

Nanomaterials (NMs) have been receiving increased attention in both academic and industrial contexts. Their particular properties, which are a consequence of their small size, present a huge potential for applications in a wide range of areas such as electronics, biomedicine, personal care products, among many others. This has been driving an increase in NM manufacture, either for academic research or for industrial and commercial applications. As their manufacture increases, so does the probability of exposure to these materials. Simultaneously, the awareness about the presence of microplastics (MP) in the environment is raising as well. MP result from the degradation of larger plastic items after prolonged exposure to, for example, ultra-violet radiation and physical abrasion. While an extensive knowledge exists about the toxic potential of macro-sized plastics, MP are only recently beginning to gain attention from regulatory entities. Both NMs and MP present a considerable toxic potential that is still largely unexplored. The nano-size of NMs could confer them the ability to bypass many biological barriers, infiltrate and disrupt sub-cellular elements, interact with several cellular processes or cause other types of detrimental effects still unknown. Some of these phenomena have already been reported, mainly in *in vitro* models or *in vivo* mammalian models. MP have also been shown to interfere with living organisms. The accumulation of ingested MP in the digestive tract of several marine organisms has been reported, even though the effects of such accumulation remain largely unknown.

During the present work, the toxic potential of gold nanoparticles (AuNP) and polyethylene MP was evaluated in the freshwater cladoceran *Daphnia magna*. This organism was chosen because it is one of the most widely used model organisms in ecotoxicology research, as it is considered representative of freshwater zooplankton and primary freshwater consumers. To achieve this, a chronic ecotoxicological bioassay was conducted with AuNP, MP and mixtures of both types of particles. In a first phase, the methodologies associated with *D. magna* culture maintenance were learned and trained. This included not only the handling and maintenance of the animals, but the maintenance of *Chlorella vulgaris* cultures as well, as these microalgae were used to feed the daphnids throughout the duration of the work. The second phase consisted on the characterization of the AuNP and MP. Calibration curves were determined and degradation assays were conducted in ultra-pure water and *D. magna* culture medium, using spectrophotometry or spectrofluorimetry. Finally, the bioassay to access chronic toxicity in *D. magna* was conducted, using reproduction as the effect criterion. The bioassay had a duration of 21 days, during which animal mortality and reproduction parameters were monitored daily. The null hypotheses tested were: i) AuNP at concentrations in the low ppm are not toxic to *D. magna*;

ii) MP at concentrations in the low ppb are not toxic to *D. magna* and iii) the presence of MP in the water does not influence the toxicity of AuNP to *D. magna*. The obtained results suggest that both AuNP and MP alone can induce toxicity in *D. magna*, causing mortality and reducing reproductive fitness of the exposed individuals. It was also observed that AuNP and MP, when in mixture, appear to interact with each other, intensifying the toxic potential observed in *D. magna*. Therefore, all three null hypotheses tested were rejected.

Keywords: *Daphnia magna*, chronic toxicity, gold nanoparticles, microplastics, mixture toxicity, nanotechnology.

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Abbreviations List

ANCOVA	-	Analysis of Co-Variance
1-ANOVA	-	One-Way Analysis of Variance
2-ANOVA	-	Two-Way Analysis of Variance
ANOVA	-	Analysis of Variance
ASTM	-	American Society for Testing and Materials
AuNP	-	Gold Nanoparticle
C. Vulgaris	-	*Chlorella vulgaris*
CI	-	Confidence Interval
CTAB	-	Cetyltrimethylammonium Bromide
D. magna	-	*Daphnia magna*
EC	-	Effective Concentration
FI	-	Fluorescence Intensity
LC	-	Lethal Concentration
LOEC	-	Lowest Observed Effect Concentration
EPA	-	Environmental Protection Agency
MBL	-	Marine Biological Culture
MP	-	Microplastic
MPA	-	Mercaptopropionic Acid
NM	-	Nanomaterial
NOEC	-	No Observed Effect Concentration
NP	-	Nanoparticle
OD	-	Optical Density
OECD	-	Organisation for Economic Co-operation and Development
PAH	-	Polyallylamine Hydrochloride
PMMA	-	Poly(methylmethacrylate)
ROS	-	Reactive Oxygen Species
UV	-	Utraviolet
Uv-vis	-	Ultraviolet-Visible

1. Introduction

1.1 Nanotechnology

Nanotechnology is a multidisciplinary science that studies the properties, manufacture, applications and interactions of systems and materials at the nano ($<10^{-9}$ m) scale. According recent definitions, a nanomaterial (NM) is a structure that has at least one dimension between 1 and 100 nm (Nel *et al.*, 2006). Due to their small size, NMs exhibit interesting properties that are not found in their corresponding bulk counterparts (Nel *et al.*, 2006) that could be applied to create new products or to enhance and update existing products in numerous industrial, academic and personal contexts. Even though the concept of "nanotechnology" is a recent one, there are some known uses of NMs throughout the human history. One of the most famous examples of NM usage in ancient times, albeit probably an unintentional one, is the Lycurgus Cup, a roman chalice with gold and silver nanoparticles (NPs) dispersed throughout the glass. The cup appears to be red when lit from behind or green when lit from the front as a result of the NPs' different absorption and reflection properties (Freestone *et al.*, 2007). However, it was only in 1857 that Michael Faraday described for the first time how the nano size of gold particles in colloidal gold suspensions influences their behavior and macroscopic properties (Faraday, 1857). NMs can have natural or anthropogenic origins (Dhawan and Sharma, 2010). Some examples of natural sources of NMs include volcano fumes, forest fires, dust storms and viruses (Navarro *et al.*, 2008; Doak *et al.*, 2009). Anthropogenic NMs can be of primary origin or secondary origin. Primary origin NMs are those that are designed and manufactured to be used in academic or industrial context, while secondary origin NMs are accidentally generated by human activity, mainly air pollution and waste degradation (Doak *et al.*, 2009). Current technology allows for the creation and manipulation of structures at the molecular and atomic scale (Singh *et al.*, 2011). In recent years, the volume of anthropogenic NMs has been increasing (Navarro *et al.*, 2008), both in quantity as well as in diversity. It is estimated that hundreds of different NMs have already been developed (Doak *et al*., 2009) in a wide selection of sizes, shapes, compositions and surface modifications. The nanotechnology industry is expanding every year, with new NMs being developed at a fast rate (Doak *et al*., 2009). This increase in NMs manufacture is motivated by the enormous potential for applications, which

brings not only academic interest but also investments in the field of nanotechnology.

1.1.1. Materials' Properties at the Nano Scale

It is thought that at the nano scale, quantic interactions prevail over the conventional chemical interactions (The Royal Academy of Engineering, 2004; Handy *et al.,* 2008) conferring the NMs their particular optical, magnetic and electric properties. Their small size results in an increase of the surface area to volume ratio, meaning that for the same volume of material, NMs offer a higher surface area compared to bulk materials (Nel *et al*., 2006). This increases the amount of atoms and molecules readily available to interact with the particles' surroundings and makes NMs highly reactive (Nel *et al.,* 2006). It is also possible that, because of this, they adsorb and concentrate high quantities of surrounding substances, modifying their bioavailability (Handy *et al.,* 2008; Navarro *et al*., 2008). Their small size means that NMs might be able to interact with biological systems in unpredictable ways (Doak *et al.,* 2009). There might exist several physical and chemical factors that, while not relevant to conventional toxicology studies, could be crucial to address in nanotoxicology studies. Therefore, it's imperative to adapt current methodologies and experimental designs in order to account for their unique properties (Doak *et al*., 2009).

One step that must be present in every nanotoxicology study is particle characterization (Burleson *et al.,* 2004). This can be achieved through different methods and techniques. It is often advisable to use multiple methods of characterization, as each method provides different and complementary types of information, which can be useful to later interpret the obtained results (Cho *et al.,* 2013). Particle characterization is also important to allow the comparison between different studies and to facilitate the consolidation of knowledge as new studies are published. The most frequently used methods for characterization of NPs include ultraviolet–visible spectroscopy (UV-Vis) to determine NP concentration and size (Haiss *et al.,* 2007), dynamic light scattering to determine the particles' zeta potential, surface charge and hydrodynamic diameter (Cho *et al.,* 2013), transmission electron microscopy to evaluate the particles' shape (Wang, 2000) and X-ray photoelectron spectroscopy to determine the particles' surface composition (Zhang *et al*., 2004).

Some types of NPs are prone to form aggregates in aqueous solutions (Handy *et al.*, 2008), so another important aspect to consider is their aggregation state and size distribution (Navarro *et al.,* 2008). Zeta potential measures the electrostatic potential of a particle at the double layer relative to the bulk fluid away from the particle and is an indicator of the stability of a NP solution (Patil *et al*., 2007). As the absolute value of the zeta potential approaches zero, the stability of the solution decreases and the tendency to form aggregates increases (The Royal Academy of Engineering, 2004; Navarro *et al*., 2008). The most common method to control NP aggregation is to use ultrasounds to disperse the particles (Bihari *et al*., 2008). It has been shown that the tendency of NPs to form aggregates varies with the pH and ionic force of a given solvent (Navarro *et al*., 2008) so, alternatively, changing the solvent might also work, but this may also alter other properties of the NP solution. A factor that can significantly alter NPs properties and behavior is their external coat (Christian *et al.,* 2008). This can modulate the particles' surface charge, total size (as opposed to the core size), reactivity, stability (Navarro *et al.,* 2008) and determines the nature of the interface between the NPs and their surrounding environment (Christian *et al.,* 2008). Some NPs are relatively easy to coat and can be modified in order to give them new properties and functionalities (Ghosh *et al.,* 2008; Zhou *et al*., 2010). These properties give NMs a wide range of potential applications, as well as new challenges in understanding their mechanisms of action, toxicity and interactivity with other materials, biological systems and ecosystems.

1.1.2. NMs' Applications

The nanotechnology industry is expected to continue growing and expanding its range of applications in several areas. The properties of NMs open new opportunities to design completely new products, processes and techniques or to significantly improve upon those that already exist. Some areas that could benefit even more from nanotechnology include:

- **Medicine:** NMs can be used as both diagnostic and treatment options in a variety of conditions and procedures. For example, iron oxide NPs are already being used as contrast agents for magnetic resonance imaging (Doak *et al.*, 2009), allowing for a better contrast with lower concentrations of the contrast agent. Silver NPS are known to have antibiotic activity and could be applied in catheters to lower

the risk of infections (Samuel and Guggenbichler, 2004); silver NPs are already being used in wound dressings and surgical instruments with the same purpose (The Royal Academy of Engineering, 2004; Chen and Schluesener, 2008). NPs are also being studied as targeted drug delivery agents (Oberdörster *et al*., 2005), NP arrays could find use in new detection and diagnostic tools (Pattani *et al*., 2008; Pek *et al*., 2008) and nanoporous scaffolds show promising results in bone tissue regeneration (Pek *et al.,* 2008).

- **Environmental remediation:** some NPs are capable of absorbing large quantities of inorganic contaminants, making them useful for both *in situ* and *ex situ* soil and water remediation (Zhang, 2003). Organic pollutants can also be addressed using highly reactive NPs that are capable of degrading them, mainly by promoting photooxidation (Tratnyek and Johnson, 2006). For example, gold NPs (AuNP) can be used to selectively remove mercury from waters containing multiple pollutants (Ojea-Jiménez *et al.,* 2012), and iron oxide NPs are being studied to be used for soil remediation of arsenic (Shipley *et al.,* 2011). Polymeric NPs can be used to enhance the *in situ* biodegradation rate of organic pollutants such as phenanthrene and other polycyclic aromatic hydrocarbons (Tungittiplakorn *et al.,* 2005).

- **Electronics:** the first piece of nanoelectronic technology was a field effect transistor measuring less than 100nm, designed and manufactured in 2000 (Gargini, 2004). Nowadays, several common devices such as computer processors or memory chips include nanoelectronic technology. More recently, quantum dots are being studied as light emitting diodes (LED), presenting advantages over organic LEDs such as their thermic stability and pure emission color (Molaei *et al.,* 2012). The electronic and optical properties of carbon nanotubes are being studied for applications in photovoltaic cells (Wang *et al.,* 2015).
- **Personal care products:** NPs are already used in cosmetics and personal care products. For example, titanium (TiO_2) and zinc (ZnO) NPs are used in some sunscreens due to their high ultraviolet (UV) absorption and low reactivity with the human skin (Nel *et al.,* 2006). Silver NPs are being incorporated in underarm deodorants due to their antibacterial properties (Raj *et al.,* 2012), and

nanocapsules - NPs containing an active ingredient on their interior - are being used to overcome some problems in cosmetic dermatology, such as the incompatibility of different ingredients used in creams and gels (Morganti, 2010)

This wide range of application fields is driving an increasing interest and investments on nanotechnology (Nel *et al.,* 2006). However, knowledge about their toxicological potential lags behind their development and application, and the consequences of acute or chronic exposure of humans, wildlife and ecosystems to NMs are still largely unknown, despite the increasing number of studies that have been made in the last years focused in a relatively reduced amount of substances in a limited variety of species and biological systems.

1.2. Gold Nanoparticles

AuNP, also known as colloidal gold, are one of the most studied NMs (Eustis and El-Sayed, 2006; Khlebtsov and Dykman, 2011) mainly due to their presumed biocompatibility and unique optical and electronic properties (Eustis and El-Sayed, 2006). In recent years, AuNP have been receiving increasing attention, with the number of publications relating to these particles rising exponentially every year (Eustis and El-Sayed, 2006). These particles are included in the "List of Representative Manufactured Nanomaterials" published by the OECD in 2010, which includes manufactured NMs that are already on the markets or are predicted to be commercialized soon (OECD, 2010). AuNP already find applications in several areas of physics and chemistry (Daniel, 2004), but recently there has been an increasing interest in exploring their potential biomedical applications (Dykman and Khlebtsov, 2012). AuNP can be modified with several surface ligands (Sperling *et al.,* 2008), allowing them to be functionalized with biologically active molecules such as antibodies, providing them with precisely tuned targeting (*Dreaden et al*., 2012). This makes AuNP very interesting from a therapeutic point of view, as they could become a "magic bullet" type of drug delivery agent, targeting exclusively the cells or tissues of interest (Pissuwan *et al.,* 2011; Kumar *et al.,* 2013). Furthermore, their small size could allow for the delivery of molecules in biological sites that remain inaccessible to current drug delivery agents (Jong *et al.,* 2008). Biodistribution studies with mice have shown that NPs pass through several biological barriers and tend to accumulate on the

liver and spleen, but smaller particles can be found in other places such as the heart, testis, lungs and even in the brain, passing through the blood-brain barrier (Jong *et al.*, 2008; Sonavane *et al.*, 2008, Lasagna-Reeves *et al.*, 2010). It is also known that AuNP are capable of converting light into heat - a phenomenon called "photothermal effect" (Huang and El-Sayed, 2011). In order to explore this property, some studies have been emerging about the usage of AuNP as agents for targeted thermal ablation of cancer cells (O'Neal *et al.*, 2004, Letfullin *et al.*, 2011). AuNP are also being studied as miniaturized biological sensors, being capable of detecting oligonucleotides, proteins and other biomolecules (Saha *et al.*, 2012). This can be used to create new diagnostic tools. Their photophysical properties can also be exploited to enhance bioimaging techniques (Dreaden *et al.*, 2012). Furthermore, AuNP have a low environmental background, making them easy to track within a living organism (Skjolding *et al.*, 2014) and are resistant to dissolution in typical environmental and biological conditions (Bozich *et al.*, 2014), which facilitates their study in biological systems.

1.3. Microplastics

Ever since they began being mass produced during the 1940s (Cole *et al*., 2011) plastics production has been growing at a fast pace every year (Fossi and Depledge, 2014). Due to the wide diversity of plastic polymers, they have been introduced in nearly all contexts, from household items, construction materials, several industries and even medical devices (Andrady and Neal, 2009). Reports show that 311 million tonnes were manufactured worldwide in 2014, accounting for 4-6% of all oil production (Plastics Europe, 2015). Polypropylene, polyethylene, polyvinyl chloride and polyurethane were the plastic types with greater demand in Europe in 2014, accounting for 66.3% of the total demand (Plastics Europe, 2015). Plastic items are considered to be safe for human health as they are largely biologically inert and non-toxic (Lithner, 2011). However, the presence of additives, catalyst remnants or polymerization solvents that remain in the plastic items after their manufacture can become problematic in certain biological contexts. One of the major reasons that make plastics such a widely used material is not only their versatility but also their resistance to degradation (Fossi and Depledge, 2014). However, their resistance to degradation is also the main reason why plastics pollution is becoming an increasing problem as they are able to persist in the environment during

thousands of years (Barnes *et al.*, 2009). Their persistence in the environment causes larger plastic items to slowly degrade into micro sized particles (Barnes *et al.*, 2009) commonly referred to as “microplastics” (MP). This degradation can be the result of prolonged exposure to UV radiation, heat, oxidation, ionic radiation as well as physical abrasion (Lithner 2011; Besseling *et al.*, 2014).

1.4. Nanotoxicology

Nanotoxicology presents a new challenge as the study of NMs has some particularities that must be addressed in order to accurately study their toxic potential. Rather than their mass, is thought that particle size and surface area are the main factors that modulate the NMs' toxic potential (Handy *et al.*, 2008). This creates doubts when trying to define the best metric to assess what constitutes a "dose" of NMs. This means that nanotoxicology might not be as straightforward as classical toxicology, as the dose-response dynamics are probably more complex to define and understand (Elsaesser and Howard, 2012).

NMs not only show properties considerably different from their bulk counterparts, but their fate and toxicity in organisms and ecosystems can also change drastically among NMs with the same composition but with different sizes, coatings and surface charges (Elsaesser and Howard, 2012). Because of this, NM interactions with living organisms are very difficult to predict or to extrapolate from other nanotoxicity studies, even when the particle composition and size are similar (Ginzburg and Balijepalli, 2007; Handy *et al.*, 2008; Elsaesser and Howard, 2012). In addition, a considerable lack of adequate and standardized methodologies for NM toxicity testing exists (Dhawan and Sharma, 2010, Khlebtsov and Dykman, 2011). Considering the wide use that some NMs already have and the expected increase of NMs use, including new substances, in the next future, both technology for toxicity testing and knowledge on the toxic effects of NMs are urgently needed to improve the bases for human and environmental risk assessments. Unlike most other materials, due to their small size, NMs might be able to penetrate biological barriers and easily gain access to the intracellular environment (Lynch *et al.*, 2007). It has already been shown that NPs are capable of crossing the bilipidic cellular membrane (Verma and Stellacci, 2010; Yacobi *et al.*, 2010) either by simple diffusion of the particles through the biological barrier or by active transportation through endocytosis (Nabiev *et al.*, 2007;

Sengstock *et al.*, 2011; Pietroiusti *et al*., 2013). It is also possible that NP entrance follows the disruption of cellular membranes. Cellular barriers are crucial in order to maintain homeostasis and intracellular compartmentalization of the different organelles. NPs might cause direct physical damage to the membranes or cause lipid oxidation (Kruis *et al.*, 1998, Handy *et al.*, 2008), possibly leading to severe impairment of cellular function or even death. After being internalized by a living organism, NPs can induce a variety of chemical or physical responses. It is thought that reactive oxygen species (ROS) production (Xia *et al.*, 2006; Auffan *et al*., 2008; Gou *et al.*, 2010), release of toxic ions (Kittler *et al.*, 2010), lipid peroxidation (Kamat *et al.*, 2010), protein folding modifications (Xia *et al.*, 2008), genotoxicity (Gou *et al.*, 2010) and disruption of biological membranes (Handy *et al*., 2008; Gou *et al.*, 2010) are among the effects that NPs can induce once inside a cell. Potential subcellular targets of NP toxicity include the mitochondria, lysosomes, cellular membranes, nucleus (Williams *et al.*, 2009; Sengstock *et al*., 2011), golgi apparatus or other vesicles (Foley *et al*., 2002; Sengstock *et al.*, 2011; Elsaesser and Howard, 2012).

However, NPs rarely exist in the environment as naked (without any coat) particles. Most NPs are either manufactured with a known coating or immediately adsorb other particles from their surrounding environment (Lynch *et al.*, 2007). In biological context, this means that several biological molecules can adsorb to the NPs, forming what is known as a corona - a layer of proteins, lipids or other biological molecules adsorbed strongly or weakly to a particle. It is that external corona that interacts with the NPs' surrounding environment, effectively "camouflaging" them in biological context (Lynch *et al.*, 2007), further granting them access to subcellular locations otherwise inaccessible. This phenomenon can also cause protein unfolding or fibrillation at the NP surface (Xia *et al*., 2008), with consequent loss or modification of protein activity. The presence of coats or a corona can modify the particles' toxic potential (Lundqvist *et al.*, 2008). Surface charge, catalytic properties and aggregation potential are among the properties that can vary widely with different coatings, core composition notwithstanding (Christian *et al.*, 2008, Navarro *et al*., 2008).

1.5. Toxicity Assessment

As the nanotechnology field expands its applicability, so does the probability of

exposure to NMs, and the necessity to assess their toxic potential becomes evident. Toxicity and ecotoxicity studies are crucial in order to understand how different substances (either naturally occurring or man-made) interact with living organisms. The information generated during these studies can then be used by governmental institutions to regulate manufacture, distribution and disposal conditions of chemical substances (Zbinden and Flury-Roversi, 1981). Generally, toxicity testing is designed around the principle that, all other factors being equal, the response of living organisms to a given substance is dependent on the dose or the concentration to which the organisms are exposed (Klaassen, 2013). From this principle, a dose-response (or concentration-response) relationship can be obtained by plotting the values of the concentration of the studied substance against the measured effect (the response being studied). To aid the organization and consolidation of knowledge generated through different laboratories, as well as to increase the replicability of the results, a number of organizations developed standardized test methods. The Organisation for Economic Co-operation and Development (OECD), the American Society for Testing and Materials (ASTM) and the United States' Environmental Protection Agency (EPA) are some entities that have published several documents describing standardized methodologies for toxicological testing of chemical substances.

During acute toxicity testing with aquatic organisms, the response under study often is the survival of the individuals exposed to the toxic agent during a short period of their life cycle through the water (Klaassen, 2013). From the toxicity curve obtained (probit transformed percentage of mortality *versus* log of the chemical concentration), the median lethal concentration (LC_{50}) can be estimated (ASTM, 1980). This is the concentration of the chemical agent estimated to induce 50% of mortality on the population tested in the specific conditions used (Klaassen, 2013). If an effect criteria other than mortality is used, the ecotoxicological parameter to be estimated is the median effective concentration (EC_{50}). From the toxicity curve other LC and EC concentrations can be estimated, such as LC_{10} or EC_{10} (ASTM, 1980). Ecotoxicity chronic toxicity testing aims at assessing the effects induced by relatively low concentrations of a chemical substance over a longer period of time than in acute toxicity testing. In general, this period should cover a considerable part of the life cycle or at least particularly sensitive stages (ASTM, 1980). It is often useful to determine the NOEC (No Observed Effect Concentration - the highest concentration for which there is no statistical

significant differences relatively to the control group) or the LOEC (Lowest Observed Effect Concentration - the lowest concentration for which there are statistical significant differences relatively to the control group) for a given substance under specific testing conditions. The most common effect criteria are somatic growth and reproduction reduction, and/or decrease of the population growth rate (ASTM, 1980). However, other effect criteria may be used, such as morphology, behavior, enzymatic activities and other sub-individual alterations, and/or other relevant endpoints.

Throughout the duration of both acute and chronic bioassays it is possible that the toxic agent being studied changes some of its properties over time and it's important to monitor those changes and how they can affect the obtained results (Meent 2007). Degradation, volatilization, sedimentation and adsorption of the toxic agent to the test beacker are some examples of phenomena that can happen over time and that should be taken in consideration during the bioassays (Meent, 2007; la Farré *et al.*, 2008). When using standard toxicity testing guidelines (e.g. OECD guidelines for chemical toxicity testing), in general the concentration of the tested substance should be maintained constant and its degradation should not exceeded a certain value (e.g. 80%) (OECD, 2012). A strategy that can be used in order to minimize this problem is to design the assay as a semi-static renewal test or to use a flow-through method of medium renewal (OECD, 2004). During semi-static renewal tests, the testing medium is renewed periodically, either by moving the test organisms to a new test-chamber with freshly prepared medium of by removing the medium in the test-chamber and replacing it with freshly prepared medium (Meent, 2007). The periodicity of the medium renewal should be determined in accordance with the known degradation rate of the chemical being tested. If this information is not known, degradation assays should be conducted prior to the toxicity tests. The flow-through method is ideal in order to assure that the concentration of the tested chemical remains constant over time but requires a large volume of test solution and generates more toxic residuals (Meent, 2007). With this method, the test chamber is continuously provided with new test medium, keeping the test concentration stable throughout the duration of the assay.

1.6. *Daphnia magna* as Model Organism

A significant part of toxicology research relies on the study of model organisms and how potentially toxic substances interact with them (Nikinmaa, 2014). In Ecotoxicology, often a good model organism to use in laboratory bioassays must fulfil some criteria such as having a reasonable size, be relatively easy to maintain in laboratory conditions and have a short generation time (Nikinmaa, 2014). It is also of crucial importance to have in-depth knowledge about their anatomy, physiology, life-cycle and ecology. Only then it becomes possible to understand how any findings done of a particular model organism can be generalised and what broader conclusions can be derived from those findings (Nikinmaa, 2014).

Daphnids, commonly known as "water fleas", are a genus of fresh-water crustaceans that have been used in biology research for a long time, dating back to 1933 (Martínez-Jerónimo, 1994). They are small filter-feeders that usually live in lakes and ponds and have a cyclical parthenogenetic reproduction cycle (Lampert, 2006). *Daphnia magna* is one of the most readily available species for ecotoxicology research (Mark and Solbé, 1998) and a large volume of publications relating to this species exist (Martínez-Jerónimo *et al.*, 1994) because it was one of the first organisms used in aquatic toxicity testing and is one of the most used species. *D. magna* exhibit several characteristics that fulfil the aforementioned criteria for good model organisms in ecotoxicology research (Pérez and Beiras, 2010). For example, they are small in size, reaching 5 mm maximum (Ebert, 2005), can be cultivated in simple media and can easily be kept individually in small flasks or in group cultures with a high number of individuals in larger recipients (Martínez-Jerónimo *et al.*, 1994; Lampert, 2006). Their life cycle is relatively short, and they reproduce by cyclic parthenogesis (Shaw *et al*., 2008). Neonates are born as juveniles, which are identical to an adult daphnid, but smaller in size (Ebert, 2005). The adult phase begins when the first eggs are laid into the brood pouch, marking the start of their reproductive cycle (Kee and Ebert, 1996). Under favourable conditions they reproduce by parthenogenesis, originating a population of almost genetically identic females over several generations (Decaestecker *et al.*, 2009), usually known as clones. Under adverse conditions, females produce two haploid resting eggs instead of a large quantity of parthenogenetic diploid eggs. These resting eggs are encapsulated by a melanised protective structure called the *ephippium* (Ebert, 2005) and must be fertilized

by a male. These eggs are extremely resistant to adverse conditions and can remain viable up to 150 years (Decaestecker *et al.*, 2009). *D. magna* is known to be highly sensitive to slight variations of their environmental water - such as the introduction of a test substance (Mark and Solbé, 1998) - and to substandard food items (Martínez-Jerónimo *et al.*, 1994). Their diet consists mainly of planktonic algae, but other particles such as bacteria can also be caught by their filtering apparatus (Ebert, 2005). *D. magna* in general plays an important role in the freshwater ecosystems where it occurs, as primary consumer. It preys over the phytoplankton, preventing algal blooms and contributing to water clearance, and is an important prey for higher level predators (Lampert, 2006). *D. magna* is widely spread across different geographical areas worldwide and it's considered representative of primary freshwater consumers and zooplankton (Lampert, 2006).

1.7. Toxicological Potential of AuNP

The knowledge about the potential toxicity of AuNP in *D. magna* is still extremely limited. Wray *et al.* studied the uptake and depuration rates of AuNP with different sizes (6nm, 20nm and 30nm) and concluded that the main factors contributing to the total body burden of NPs in exposed daphnids were the core size and surface charge (Wray and Klaine, 2015). Skjolding *et al.* arrived to the same conclusion while studying 10nm and 30nm AuNP with two different coatings (mercaptoundecanoic acid and citrate) but noted that size and surface charge seemed to be inconsequential for the depuration of NPs from the organism (Skjolding *et al.*, 2014). This seems to be related to other studies that compared AuNP with different coatings and surface charges. Bozich et al found that positively charged AuNP (cetyltrimethylammonium bromide (CTAB) and polyallylamine hydrochloride (PAH) coatings) were significantly more acutely toxic to *D. magna* than negatively charged (citrate and mercaptopropionic acid (MPA) coatings) AuNP. Chronic toxicity also varied with the different coatings, but didn't seem to be related to the particles surface charge. The authors noted that negatively charged AuNP were less stable and would form aggregates easier, which might be related to their acute toxicity (Bozich *et al.*, 2014). Dominguez *et al.* used AuNP with the same coats (MPA, Citrate, PAH and CTAB) and sizes as the aforementioned study during 24 h acute toxicity essays with adult *D. magna* and observed that positively charged coats induced elevated levels of ROS in the gastrointestinal tract and caused an overexpression of 4 genes

associated with oxidative stress compared with negatively coated AuNP and with the control group (Dominguez *et al.,* 2015). Other group reported that gene expression analysis suggested that PAH-coated AuNP could induce toxicity through damage in the cytoskeleton during 21-day chronic exposure times (Qiu *et al.,* 2015).

It is possible that AuNP are ingested and accumulate in *D. magna*'s guts. Lee at al. observed that 21 nm citrate stabilized AuNP seemed to decrease their concentration in the testing media in the presence of *D. magna* juveniles after 12 h of exposure (Lee and Ranville, 2012), which suggests that the AuNP were internalized by the living organisms. Once ingested, AuNP were found by numerous studies to accumulate in the daphnids gut (García-Cambero *et al*., 2013; Gilroy *et al.,* 2014; Khan *et al*., 2014; Skjolding *et al*., 2014). *D. magna* is only capable of filtering particles in the size range of 0.4-40 μm (Baun *et al.,* 2008) and smaller particles such as NPs should not be ingested by the daphnids. However, not only are NPs internalized, but smaller particles could have higher uptake rates than larger particles, as was observed in a study by Wray *et al.* where 6 nm particles had higher uptake and depuration rates that 20 nm and 30 nm AuNP (Wray and Klaine, 2015). There isn't yet a clear explanation for these observations, but a hypothesis is that these NPs could be internalized by two pathways: passive diffusion of smaller particles through biological membranes or active ingestion of aggregates by filtration. AuNP aggregation seems to be a common occurrence and can happen very soon (<1 h) after the addition of the particles to the testing media (García-Cambero *et al.,* 2013; Gilroy *et al.,* 2014; Skjolding *et al*., 2014; Lee *et al.,* 2015). After being internalized, AuNP seem to induce toxic effects on the exposed organisms. Naked AuNP with a diameter of 21 nm exhibited a EC_{50} of 70 mg/L after 48 h of exposure with *D. magna* juveniles while ionic gold (in the form of aurochloric acid) had, for the same exposure time, as EC_{50} of 2 mg/L (Li *et al.*, 2010). As for chronic exposure, after 8 days of exposure to naked 21 nm AuNP at 10 mg/L concentration no effects were found on *D. magna* birth rates or embryo development (Li *et al.,* 2010). However, the exposed individuals molted more often and had a shorter life span comparing to the control group. These results show that AuNP are capable of being internalized by *D .magna* and can cause acute and chronic toxicity in these organisms.

1.8. Toxicological Potential of MP

It's been shown that plastics have been accumulating on aquatic environments such as oceans and rivers over the years (Barnes *et al*., 2009; Wright et al, 2013b) as a result of the increase in their usage and disposal. Although there is an abundance of studies and reports about the impact of macro-sized plastic items in the environment, micro-sized plastics are only recently gaining the attention of academic and regulatory entities (Barnes *et al*., 2009). Because of their small size and consequent ability to evade sewer filtration facilities (Cauwenberghe *et al*., 2013), it's assumed that MP exist in aquatic environments in significant amounts and that their presence can induce toxicity in several aquatic organisms (Galgani *et al*., 2014). Even though most research is focused on the MP presence and effects in marine environments, an increasing number of publications have been emerging about their interactions with freshwater ecosystems. In recent years, the presence of MP in freshwater environments has become evident. Imhof *et al.* reported that low density MP (mainly polystyrene and polyethylene) were found in the beach sediments of Lake Garda, one of the main tourist spots in Italy (Imhof *et al*., 2013). In the same country, other authors found significant amounts of micro-sized polyethylene and polypropylene fragments in ten different samples of sediments from the lagoon of Venice (Vianello *et al.,* 2013). MP were also found in St. Lawrence River (Canada) (Castañeda *et al*., 2014) and in an urban river in Chicago (United States of America) (McCormick, 2014). Eriksen *et al*. studied the presence of MP along the Laurentian Great Lakes (United States of America) and detected a sharp increase in MP abundance on the samples collected in a location downstream from two major cities (Eriksen *et al*., 2013), showing a clear correlation between human population density and MP abundance in the environment. This was also shown during a study conducted by Free *et al.*, where MP density decreased with the distance from the most populated areas along Lake Hovsgol (Mongolia) (Free *et al.,* 2014). The main sources of MP are thought to be the degradation of larger plastic items (Besseling *et al*., 2014) that reach the environment through incorrect and uncontrolled disposal of plastic consumer items (Hopewell *et al.,* 2009) or industrial processing plants that remain unregulated due to a regulatory gaps regarding MP handling and disposal (Lechner and Ramler, 2015).

Plastic microbeads have been used in feeding research with filter feeders such as sponges or some copepods such as *Eurytemora affinis* (Powell and Berry, 1990). More

recently, it has been found that MP are capable not only of being ingested, but also accumulate rapidly on the digestive track of freshwater filter feeders such as the blue mussel (*Mytilus edulis*) (Browne *et al.,* 2008). This raises concerns about the potential toxic effects that these particles might induce after ingestion by freshwater organisms. A particular concern related to MP ingestion is not about the particles *per se* but about their ability to adsorb and concentrate other particles and molecules in their surrounding environment. It has already been shown that MP concentrate several waterborne pollutants like metals or persistent organic pollutants such as dichlorodiphenyltrichloroethane, polycyclic aromatic hydrocarbons or polychlorinated biphenyls (Frias *et al.,* 2010). The presence of additives is yet another concern related to MP ingestion, as they can be leached out of the particles and cause several negative effects (Talsness *et al.,* 2009; Andrady, 2011). This could make the MP a vector for all sorts of contaminants, increasing their bioavailability by concentrating them and, consequently, exacerbating their toxic potential. These concerns are intensified when considering the potential accumulation and amplification of MP ingestion throughout the food web (GEF, 2012).

Regarding *D. magna*, MP could cause internal abrasion and blockades throughout the daphnids' gut or could interfere directly with the filtering apparatus (Wright et al, 2013b). Some MP are in the same size range as microscopic algae, which could cause them to be confounded as prey and to be actively ingested by the daphnids, causing a false sensation of fullness and reducing their effective feeding rates (Gophen and Geller, 1984). Rosenkranz *et al.* compared the uptake rates of differently sized polystyrene particles (20 nm and 1000 nm) by *D. magna* and reported that particle uptake and depuration was higher in mass for larger sized particles. However, when comparing the total number of particles and total surface area, the uptake of smaller 20 nm particles was significantly higher (Rosenkranz *et al.*, 2009). Booth *et al.* showed that both poly(methylmethacrylate) (PMMA) and poly(methylmethacrylate-co-stearylmethacrylate) (PMMA–PSMA) MP with sizes ranging from 86 to 125 nm are ingested by *D. magn*a after 24 h of exposure. However, only PMMA-PSMA MP induced mortality after 24h (EC_{50} = 1550 mg/L) and 48h (EC_{50} = 879 mg/L) of exposure (Booth *et al.,* 2015). Nasser *et al.* reported much lower values of EC_{50} for 80 nm polystyrene MP with two different coatings. Carboxilic acid coated plastic MP had an EC_{50} value of

36.3 mg/L for a 24 h exposure, and amino acid coated plastic MP had an EC_{50} value of 25.8 mg/L for the same exposure time. These authors also showed that pre-conditioning of the MP with proteins excreted by *D. magna* juveniles created a proteic corona that coated the MP and affected their uptake and depuration rates (Nasser and Lynch, 2015). In another study, Besseling et al reported that 70 nm polystyrene particles didn't affect *D. magna* mortality rates in concentrations up to 150 mg/L, but affected reproduction. Broods produced by exposed *D. magna* had a decreased number of neonates that were smaller in size and had higher rates of incidence of malformations. These effects were exacerbated when the MP were left for 5 days in *D. magna* culture medium with algae that were subsequently fed to the daphnids. The authors suggested that the MP could adsorb to the algae during those 5 days, which then served as a vehicle for MP delivery and increased the total number of MP ingested (Besseling *et al*., 2014). This could be a significant factor that is currently being overlooked, as in real-life scenarios MP are sure to interact with a variety of biological molecules that could affect their behavior and toxic potential.

The presence of anthropogenic MP in lakes and rivers is increasingly becoming clear. There isn't yet clear evidence of MP induced toxicity in *D. magna*, and there is still a gap in the knowledge about chronic exposure times to these particles. Furthermore, the fact that MP exist in the environment among many other chemicals and pollutants cannot be ignored as it can play an important role in defining the real toxic potential of MP.

1.9. Aims of the Study, Hypotheses to be Tested and Structure of the Thesis

The main goal of the present study was to assess the chronic toxicity of AuNP and MP alone and in mixture, to the freshwater cladoceran *D. magna*, using somatic growth and reproduction as effect criteria. The following null hypotheses were tested:

- H_{01}: AuNP (4-7 nm diameter) at concentrations in the low ppm range are not toxic to *D. magna*.
- H_{02}: MP (1-5 µm diameter) at concentrations in the low ppb range are not toxic to *D. magna*.
- H_{03}: The presence of MP in the water does not influence the toxicity of AuNP to *D. magna*

This thesis is structured in 4 chapters. In the first chapter, corresponding to the introduction, a short review of NMs, MP and their toxicological potential in *D. magna* is given and the hypothesis to test are proposed. In the second chapter, the materials and methodologies used during the execution of the bioassays are described. In the third chapter, the results are presented and discussed. In the fourth chapter, the hypothesis proposed are rejected or confirmed and a general overview of the work done and future prospects are presented.

2. Methodologies and Materials

2.1. *Chlorella vulgaris* Cultures

Cultures of the microalga *Chlorella vulgaris* were maintained as a food source for *D. magna*. Cultures were maintained in Marine Biological Culture (MBL) medium (Stein *et al.*, 1973) as described in Table 1 supplemented with vitamins B12, H and B1, for a maximum period of 30 days.

Table 1 - Qualitative and quantitative chemical composition for the nutrient stock solutions used for MBL culture medium.

Nutrients (Chemical Formula)	Concentration (g/L)
$CaCl_2 \cdot 2H_2O$	36.76
$MgSO_4 \cdot 7H_2O$	36.97
$NaHCO_3$	12.60
K_2HPO_4	8.71
$NaNO_3$	85.01
$NaSiO_3 \cdot 9H_2O$	28.42
$NaEDTA \cdot 2H_2O$	4.36
$FeCl_3 \cdot 6H_2O$	3.15
$CuSO_4 \cdot 5H_2O$	0.01
$ZnSO_4 \cdot 7H_2O$	0.022
$CoCl_2 \cdot 6H_2O$	0.01
$MnCl_2 \cdot 4H_2O$	0.18
$Na_2MoO_4 \cdot 2H_2O$	0.006
Buffer	Concentration (g/L)
Tris(hydroxymethyl)aminomethane	250

All chemicals used to prepare the culture media were of analytical grade and were purchased from Sigma-Aldrich (USA) or Merck (Germany). The vitamin stock solutions

were prepared in ultrapure water (MILLI-Q, Merck Millipore, Germany) and vacuum filtered with a 0.2 µm filter (Millex-GS, Merck Millipore, Germany). The prepared solutions were kept at -20ºC and thawed as needed. The nutrient stock solutions were prepared in ultra-pure water and stored at 4ºC. Culture medium was prepared by first adding the appropriate amount of each nutrient stock solution to deionized water. After the addition of the nutrients, the medium was sterilized in the autoclave (Uniclave 88, AJC, Portugal) for 60 minutes at 120ºC. The medium was left to cool off for 24 h, after which the vitamin solutions were added.

The cultures were maintained in aerated 5 L glass erlenmeyer flasks with 4 L of sterile MBL medium. The flasks were kept at 20ºC with 24h photoperiod with continuous air supply. Culture medium was partially renewed three times per week by removing 2 L of the culture and adding 2 L of fresh medium. The flasks were monitored daily to detect any signs of bacterial contamination.

D. magna food preparation was made three times per week, at the same time as media renewal. A volume of 2 L of *C. vulgaris* culture was collected and centrifuged at 3500 rpm for 7 minutes (Heraeus Megafuge 16, ThermoFisher Scientific, USA). The resulting supernatant was discarded and the pellet was ressuspended in ASTM (refer to section 2.2 for a detailed description of the ASTM medium). This algae suspension was used to feed the daphnids and was stored in a refrigerator at 4ºC for a maximum period of 3 days.

To calculate the algae density in the food preparation, a sample from the algae suspension was diluted with a factor of 1:10 (v/v) and the optical density (OD) was measured at 440 nm (V-630 Spectrophotometer, Jasco, USA). According to the obtained OD, the volume corresponding to $3.00*10^5$ cells per 100 mL was calculated using the relationship presented in Equation 1:

$$Algae\ concentration = -155820 + Abs * 13144324 * Df \quad \text{Equation 1}$$

Where:

Algae concentration = concentration of the algae at cells per mL
Abs = absorbance value of the food preparation solution at 440 nm
Df = dilution factor

2.2. *Daphnia magna* Cultures

D. magna (clone A) cultures have been maintained in the Laboratory of Ecotoxicology of ICBAS and CIIMAR for more than 15 years. The parental cultures that were used to provide the individuals used during the assays were maintained in American Society for Testing and Materials hard water (hereafter indicated as "ASTM") (ASTM, 2010) as described in Table 2, enriched with *Ascophyllum nodosum* extract (Baird *et al.*, 1989) and vitamins B1, B12 and H (Bradley *et al*., 1993).

Table 2 - Qualitative and quantitative chemical composition for the nutrient stock solutions used for ASTM culture medium.

Nutrients (Chemical Formula)	Concentration (g/L)
$NaHCO_3$	0.19
$MgSO_4 \cdot 7H_2O$	0.25
KCl	0.008
$CaSO_4 \cdot 2H_2O$	0.12

All chemicals used to prepare the culture media were of analytical grade were purchased from Sigma-Aldrich (USA) or Merck (Germany). Nutrient stock solutions were prepared in ultra-pure water and stored at 4ºC for all nutrients except for $CaSO_4 \cdot 2H_2O$, which had to be freshly prepared at the time of medium preparation. Culture medium was prepared by adding the appropriate amount of each nutrient and vitamin solutions and to deionized water.

D. magna cultures were maintained in parthenogenetic reproduction under controlled conditions inside a climate chamber (PGC 1400, Bronson, Netherlands) with

a photoperiod of 16 hours light : 8 hours dark at 20 ± 1ºC. Each individual was maintained in a 200 mL glass flask containing 100 mL of ASTM medium which was changed three times per week by moving each animal into a new identical flask with 100 mL of ASTM. The flasks were capped but not closed in order to assure oxygenation while minimizing the deposition of airborne particles in the culture medium. At the time of medium renewal, each flask was inspected and the number of juveniles per parental individual was counted. A volume corresponding to $3.00*10^5$ cells of *C. vulgaris* (prepared as described in section 2.1) was added to each flask daily. The animals were handled with a 5 mL plastic pipette with the tip cut off.

The animals were acclimatized in these conditions during at least three generations before any assay was conducted. All individuals were maintained in continuous parthenogenetic reproduction in order to reduce genetic variability during the assays. To ensure the best synchronization as possible during the assays, immediately before the start of the experiments each parental animal was monitored hourly and the neonates born during this period were pooled together in a glass flask and used to initiate the assay. Only neonates from the third to fifth brood were used during the assays.

2.3. AuNP Description

AuNP were purchased from Sigma-Aldrich (USA). The product came in the form of a colloidal suspension of AuNP in citrate buffer (0.1 mM) in a plastic bottle. The particles were described by the supplier as having a core diameter between 4 and 7 nm (±12%), a polydispersity index lower than 0.2 and an absorption maximum between 510 and 525 nm.

Characterization of AuNP was made by UV-VIS spectrophotometry, which is considered to be an adequate and cost-effective method of characterization of these particles in aqueous media (Haiss *et al.*, 2007; Amendola and Moreno, 2009; Pamies *et al*., 2014).

Colloidal solutions of 20 mg/L were prepared by dilution of the stock solution (provided by the manufacturer) in either ASTM or ultra-pure water. These solutions were then serially diluted with a 1:2 (v/v) dilution factor in ASTM or ultra-pure water to obtain

the following concentrations:

20 , 10 , 5 , 2.5 , 1.25 , 0.625 , 0.312 , 0.156 , 0.078 , 0.039 mg/L

These solutions were stored for 48h in the climate chamber used to keep the *D. magna* cultures.

Full range (200-900 nm) UV-Vis spectra for all the solutions were read with a baseline correction using ASTM or ultra-pure water, as appropriate. The readings were made at 0 h, 24 h and 48 h after preparation of the solutions. The obtained spectra were analyzed using Spectra Manager (version 2.10.01) software. This procedure was repeated 3 times, during different days, in order to obtain three independent sets of readings for each concentration and time point.

To evaluate the potential changes in the particles' size through time (hereafter indicated as "AuNP decay"), the spectra of each solution at 0 h, 24 h and 48 h were obtained. This was made to determine the most adequate time to renew the test media during the bioassays, in order to assure that the nominal concentrations of AuNP did not deviate more than 20% from the actual concentrations. The AuNP decay was calculated from the absorbance readings of the solutions at 520 nm using as indicated in Equation 2:

$$Decay\ (\%) = 100 - (abs_2 * \frac{100}{abs_1}) \qquad \text{Equation 2}$$

Where:

Decay(%) = percentage of AuNP decay compared to the absorbance reading at 0 h
Abs_2 = absorbance value at 520 nm at 24 h or 48 h, in OD units
Abs_1 = absorbance value at 520 nm at 0h, in OD units

The diameter of the AuNPs was estimated according to the method proposed by Haiss et al. (Haiss *et al.*, 2007) for gold particles with a diameter between 5 and 80 nm (Equation 3):

$$D = \exp(B1 * \frac{A1}{A2} - B2)$$ Equation 3

Where:

D = particle diameter (error ≈ 11%)

B1 = 3

B2 = 2.2

A1 = absorbance at 520nm in OD units

A2 = absorbance at 450nm in OD units

2.3.1. AuNP Calibration Curve

Initial solutions of 20 mg/L of AuNP was prepared as indicated in the previous section, in ultra-pure water or ASTM. Both calibration curves were plotted in order to determine whether it was adequate to use the calibration curve referring to the AuNP solutions prepared in ultra-pure water or if significant changes occurred in the model when the AuNP were prepared in ASTM. Standard solutions of AuNP were prepared by serial dilutions from the 20 mg/L solution with a dilution factor of 1:2 (v/v) and their absorbance at 520nm was read. The concentrations for the standard solutions were:

20 , 10 , 5 , 2.5 , 1.25 , 0.625 , 0.312 , 0.156 , 0.078 , 0.039 mg/L

The absorbance values at 520 nm were plotted against the nominal concentrations and the correlation between the variables was investigated through the Pearson's correlation coefficient. Then, a linear regression model was fitted into the data using the absorbance as the independent variable and the AuNP nominal concentration as the dependable variable, in order to calculate test concentrations of AuNP during the bioassays from the model.

2.4. MP Description

Fluorescent polyethylene microspheres were purchased from Cospheric (USA). The product came in the form of dry powder in a glass container. The particles were described by the manufactured as having between 1 and 5 um of diameter, excitation peak at 575 nm and emission peak at 607 nm, being red opaque and having a density of 1.3 g/cm^3.

In order to observe the decay of the fluorescence of the MP particles (hereafter indicated as "MP decay") through time, the emission fluorescence of each solution was read at 0 h, 24 h and 48 h after preparation of the solutions, at 575 nm for the excitation wavelength and 607 nm for the emission wavelength (FP-6200 Spectrofluorimeter, Jasco, USA). This was made to determine the most adequate time to renew the test media during the bioassays, in order to assure that the nominal concentrations of MP did not deviate more than 20% from the actual concentrations. The MP decay was calculated from the fluorescence readings of the solutions as indicated in Equation 4:

$$Decay\ (\%) = 100 - (F_2 * \frac{100}{F_1}) \qquad \text{Equation 4}$$

Where:

Decay(%) = percentage of fluorescence decay compared with the fluorescence reading at 0h

F_2 =fluorescence value at 24 h or 48 h

F_1 = fluorescence value at 0 h

2.4.1. MP Calibration Curve

Initial solutions of 12 mg/L of MP were prepared by weighting 0.0006 g (ABS 120-4 Analytical Balance, KERN & Sohn, Germany) of MP, which were then dispersed

in 0.5 L of ASTM or ultra-pure water. Both calibration curves were plotted in order to determine whether it was adequate to use the calibration curve referring to the MP solutions prepared in ultra-pure water to determine the real MP concentrations in a solution, or if significant changes occurred in the model when the MP were prepared in ASTM. Standard solutions were prepared by successive dilutions with a dilution factor of 1:2 of the initial solution. The concentrations for the standard solutions were:

12 , 6 , 3 , 1.5 , 0.75 , 0.375 , 0.187 , 0.094 , 0.047 , 0.023 , 0.012 mg/L

The emission fluorescence for each solution was read at 575 nm for the excitation wavelength and 607 nm for the emission wavelength. The fluorescence values were plotted against the nominal concentrations and the correlation between the variables was investigated through the Pearson's correlation coefficient. Then, a linear regression model was fitted into the data using the fluorescence as the independent variable and the MP nominal concentration as the dependable variable, in order to calculate test concentrations of MP during the bioassays from the model. This procedure was repeated 3 times, during different days, in order to obtain three independent sets of readings for each concentration and time point.

2.5. Bioassays

2.5.1. Test Validation with a Reference Substance

In order to assess the status of the cultures of *D. magna* and test all the procedures, an acute toxicity test was conducted with potassium dichromate ($K_2Cr_2O_7$), a reference substance, as recommended in the OECD guideline 202 (OECD, 2004). A 32 mg/L $K_2Cr_2O_7$ (Merck 104865) stock solution was prepared in ultra-pure water in a 15 mL centrifuge tube. This solution was homogenized using a vortex and was shielded from light by coating the tube externally with aluminum foil. From this solution, test solutions were prepared by successive dilutions in ASTM medium with a dilution factor of 1:2 (v/v). Thus, the concentrations tested were: 6.4 , 3.2 , 1.6 , 0.8 , 0.4 , 0.2 , 0.1 mg/L

D.magna juveniles (> 6 h and <24 h old) were individually exposed for 48 h to different concentrations of the tested chemical. Throughout the duration of the assay, the

animals were maintained in 100 mL glass flasks containing 50 mL of each test solution or ATSM (for the control group). The flasks were capped but not closed to assure oxygenation while minimizing the deposition of airborne particles in the test media. Each flask contained 5 animals and a total of 20 individuals were exposed per concentration tested. The flasks were kept in a climate chamber set to 20ºC (±1) and a light:dark cycle of 16:8 hours. The animals were not fed and the test media were not renewed during the entire duration of the test (48 h). Dissolved oxygen, temperature and pH of the test media were monitored (HQ 40d Multi-Parameter Meter, Hach, USA) at 0 h, 24 h and 48 h after the start of the test. The effect criterion was mortality, recognized by the immobilization for 15 seconds under a bright light. Immobilization was evaluated at 24 h and 48 h of exposure.

2.5.2. AuNP and MP Chronic Toxicity Assay

A chronic bioassay was carried out to assess the effects of AuNP and MP, alone and in mixture, to *D. magna*. The bioassay was carried out for 21 days, at 20ºC (±1) under a photoperiod of 16 h light : 8 h dark, following in general the OECD guideline 211 (OECD, 2012) with the adaptations required to test a mixture in full factorial design. Test medium was ASTM and the following treatments were tested: control (ASTM only), citrate-control (because the original AuNP were suspended in citrate buffer), 0.2 mg/l of AuNP, 2 mg/l of AuNP, 0.02 mg/l of MP, 0.2 mg/l of MP, 0.2 mg/l of AuNP + 0.02 mg/l of MP, 0.2 mg/l of AuNP + 0.2 mg/l of MP, 2 mg/l of AuNP + 0.02 mg/l of MP, 2 mg/l of AuNP + 0.2 mg/l of MP.

The test solutions were prepared by diluting the stock solution (provided by the manufacturer) in ASTM. A 0.1 mM solution of citrate buffer was prepared in ultra-pure water. This solution was then diluted in ASTM medium in order to match the concentration of citrate buffer present in the 2 mg/L of AuNP test solution. As for the MP, the test solutions were prepared by weighting the appropriate amount of MP and dispersing them in ASTM medium.

Glass 100 ml test beakers were filled with 50 ml of each test solution. The bioassay was started by introducing *D. magna* juveniles (> 6 h and < 24 h old) to the treatments (1 individual per test beaker, 10 individuals per treatment). They were exposed

for 21 days being feed with *C. vulgaris* every day ($3*10^5$ cells/daphnia/day), in semi-static conditions with test media renewal at each 24h. The effect criteria were somatic growth and reproduction. Daphnid immobilization was monitored daily and immobile individuals were discarded and did not count into the final results. The number of neonates (both viable and immobile) and aborted eggs was counted daily. Moulting was monitored daily. The moults were collected and used to measure the 1st exopodite from the 2nd right antennae to evaluate the size and somatic growth of each animal throughout the assay (Soares 1989). Dissolved oxygen, temperature and pH of the testing media were monitored daily. To determine the actual concentrations of AuNP and/or MP in media, samples of fresh and old medium were collected at the time of test media renewal. The actual concentrations of the tested substances and their decay in test media were determined as described in the sections 2.3 and 2.4.

2.6. Statistical Analysis

All statistical analyses were performed with SPSS Statistics package (IBM, USA) version 23. The significance level was set at 0.05 for all statistical analysis performed.

To compare the fluorescence intensity of MP in ultra-pure water and ASTM, and to compare the absorbance and diameter of AuNP in ultra-pure water and ASTM, the analysis of co-variance (ANCOVA) was used.

At the end of the acute bioassay, the percentages of mortality were calculated, probit transformed and plotted against the log concentrations of the chemical tested to obtain the toxicity curves after 24 and 48 h of exposure to the chemical. The 24 h and 48 h median effect concentrations (EC_{50}) and corresponding 95% confidence intervals were calculated from the toxicity curves.

Regarding the chronic bioassay, data concerning the individuals that did not survive until the end of the test period during the chronic bioassay were not included during the analysis. The data for each biological parameter (*i.e.* total daphnid growth, age at the first brood released, total number of broods released, total number of aborted eggs, number of immobile juveniles produced and number of viable juveniles) were tested for normality of distribution using the Kolmogorov-Smirnov test and for homogeneity of

variances (Zar, 1999) using the Lavene's test. Data transformations were done before the Analysis of Variance (ANOVA) whenever necessary. For each biological parameter, the 10 different treatments of the chronic bioassay were first compared using a one-way ANOVA (1-ANOVA) followed by the Tukey's multi-comparison test when significant differences among treatments were found by 1-ANOVA. For some biological parameters, no statistical significant differences between the control and the citrate-control groups were found. In these cases, a two-way ANOVA (2-ANOVA) with interaction was carried out, excluding the citrate-control group, to investigate interactions between AuNP and MP.

3. Results and Discussion

3.1. *Daphnia magna* Cultures

3.1.1 Number of Juveniles per Parental Individual

The average number of juveniles produced per parental animal per brood rises from the first until the 3rd brood, after which it starts declining. The average number of juveniles per brood per parental animal is presented in Table 3. The average number of total juveniles per parental animal up until the 5th brood always exceeded 60, which is the minimal number of total juveniles that each parental animal in the control group must produce in 21 days in order for a chronic assay to be valid, according to the OECD guideline 211 (OECD, 2012).

Table 3 - Average number of juveniles per animal per brood. SD = Standard Deviation

Brood number	Number of juveniles (average±SD)
1st	9 ±1.63
2nd	21 ±1.94
3rd	35 ±2.81
4th	30 ±2.05
5th	26 ±1.64

3.1.2. Acute Assay With a Reference Substance

Throughout the duration of the assay (48 h), the variation of the pH of the testing media in all the test beakers was less than 1 unit, the variation of the temperature was less than 1ºC and the dissolved oxygen remained above 3 mg/L at all times. No immobile individuals were found in the control group. The number of immobile juveniles per treatment after 24 h and 48 h of exposure is presented in Table 4.

Table 4 - Immobile animals per group after 24 h and 48 h of exposure to different concentrations of potassium dichromate.

Potassium Dichromate Concentration (mg/L)	Number of viable animals at the start of the test	Immobile animals per exposure time	
		24 h	48 h
0.0	20	0	0
0.1	20	0	1
0.2	20	2	3
0.4	20	2	3
0.8	20	4	14
1.6	20	16	19
3.2	20	19	20
6.4	20	20	20

The EC_{50} at 24h was found to be 0.987 mg/L (CI95% = 0.605-1.680), which falls inside the reference range of values (0.6 – 2.1 mg/L) provided by the OECD guideline 202 (OECD, 2004). At 48h, the EC_{50} was found to be 0.547 mg/L (CI95% = 0.427-0.707). These results indicate that the sensitivity of *D. magna* cultures is adequate and testing conditions were reliable.

3.2. Characterization of AuNP in Ultra-Pure Water and ASTM

The overall estimated diameter of AuNP in ultra-pure water and ASTM are presented in Table 5 and Table 6 and were, respectively, 5.0421 nm (±0.6324) and 4.8201 nm (±1.0496). These values are in accordance with the product description provided by the manufacturer (4 - 7 nm).

Table 5 - estimated diameter of 5 nm AuNP in solutions prepared in ultra-pure water. The values presented are the average of 3 solutions prepared independently, with the corresponding standard deviation (within brackets). Absorbance peak = absorbance at 520 nm. Overall = overall average and standard deviation. OD = Optical Density.

Ultra-pure water

AuNP concentration (mg/l)	Abs (OD units) at absorbance peak	Abs (OD units) at 450 nm	AuNP Size (nm)
0.156	0.0004 (±0.0028)	0.0018 (±0.0024)	5.273 (±1.6063)
0.312	0.0023 (±0.0013)	0.0020 (±0.0020)	5.683 (±1.3134)
0.625	0.0070 (±0.0037)	0.0053 (±0.0017)	4.833 (±0.7704)
1.25	0.0146 (±0.0050)	0.0112 (±0.0016)	5.760 (±0.1193)
2.5	0.0347 (±0.0028)	0.0280 (±0.0016)	4.565 (±1.7416)
5	0.0758 (±0.0074)	0.0596 (±0.0017)	5.063 (±0.2127)
10	0.1550 (±0.0069)	0.1248 (±0.0002)	4.605 (±0.0300)
20	0.3134 (±0.0087)	0.2530 (±0.0035)	4.555 (±0.0573)
Overall			5.0421 (±0.6324)

Table 6 - estimated diameter of 5 nm AuNP in solutions prepared in ASTM. The values presented are the average of 3 solutions prepared independently, with the corresponding standard deviation (within brackets). Absorbance peak = absorbance at 520 nm. Overall = overall average and standard deviation. OD = Optical Density.

ASTM

AuNP concentration (mg/l)	Abs (OD units) at absorbance peak	Abs (OD units) at 450 nm	AuNP Size (nm)
0.156	0.0014 (±0.0020)	0.003323 (±0.0004)	4.919 (±0.5839)
0.312	0.0031 (±0.0031)	0.002527 (±0.0011)	4.845 (±0.5832)
0.625	0.0067 (±0.0042)	0.005184 (±0.0038)	5.250 (±0.3079)
1.25	0.0135 (±0.0061)	0.010903 (±0.0045)	4.571 (±0.6499)
2.5	0.0341 (±0.0115)	0.025534 (±0.0028)	6.202 (±0.7461)
5	0.0688 (±0.0179)	0.056707 (±0.0024)	4.236 (±0.5626)
10	0.1473 (±0.0260)	0.121024 (±0.0058)	4.270 (±0.0693)
20	0.3074 (±0.0873)	0.252604 (±0.0054)	4.268 (±0.0586)
Overall			4.8201 (±1.0496)

3.2.1. AuNP Calibration Curve

Representative images of the absorption spectrum obtained from the highest (20 mg/L) concentration of AuNP prepared in ultra-pure water or ASTM are presented in Figure 1 and Figure 2, respectively. The absorption peak was found at 520 nm for both solutions, which is in accordance with the value for the commercial formulation provided by the manufacturer and with the published literature relating to 5 nm AuNP (Haiss *et al.*, 2007).

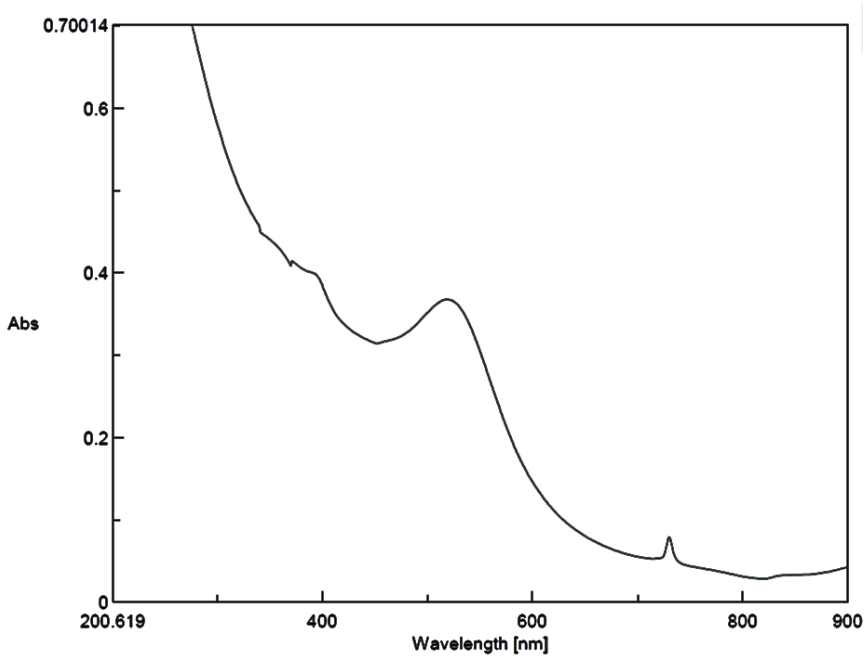

Figure 1 – Absorption spectrum of a 20 mg/L AuNP solution prepared in ultra-pure water. Abs = absorbance in optical density (OD) units. The image was obtained through the Spectra Manager software.

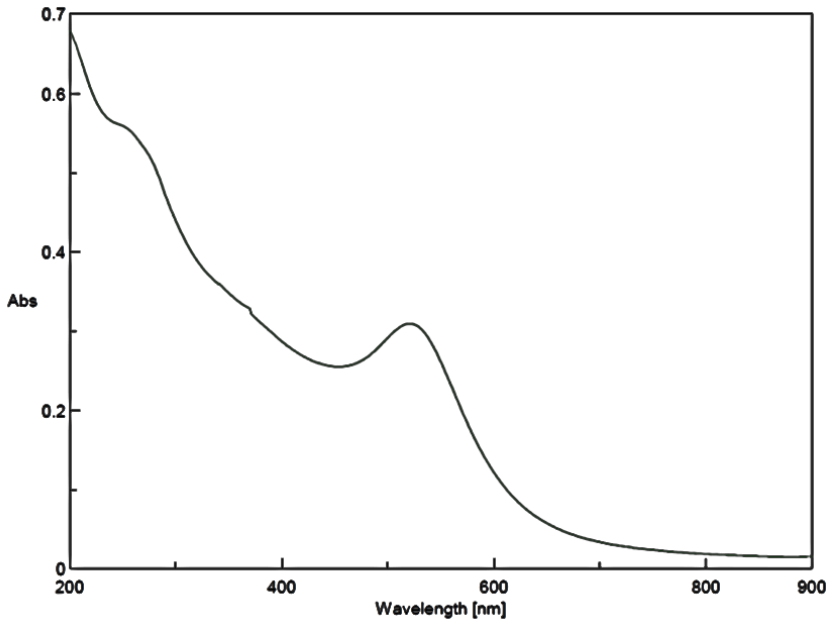

Figure 2 - Absorption spectrum of a 20 mg/L AuNP solution prepared in ASTM. The image was obtained through the Spectra Manager software. Abs = absorbance in optical density (OD) units.

For concentrations below 0.156 mg/L, as shown in Figure 3 and Figure 4, it is impossible to identify any absorption peak in both ultra-pure water and ASTM. Therefore, the calibration curves were plotted using only the values referring to solutions with AuNP concentrations of 0.156 , 0.312 , 0.625 , 1.25 , 2.5 , 5 , 10 and 20 mg/L.

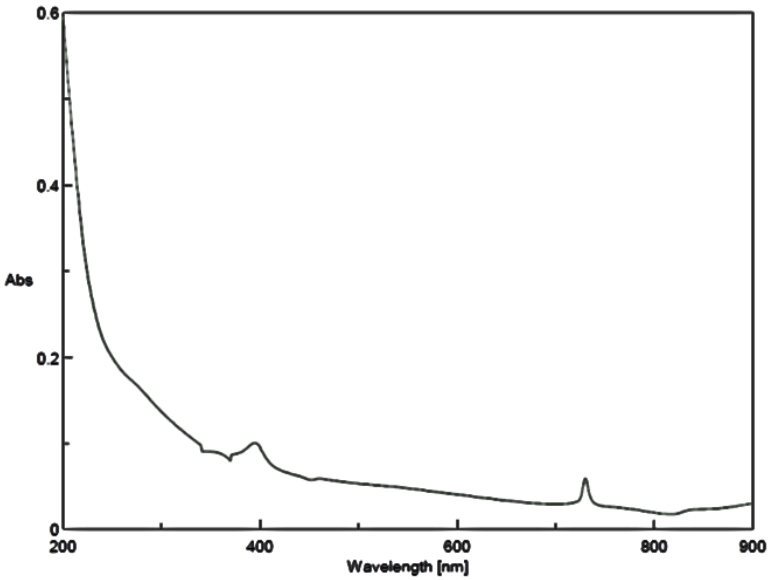

Figure 3 - Absorption spectrum of a 0.078 mg/L AuNP solution prepared in ultra-pure water. The image was obtained through the Spectra Manager software. Abs = absorbance in optical density (OD) units.

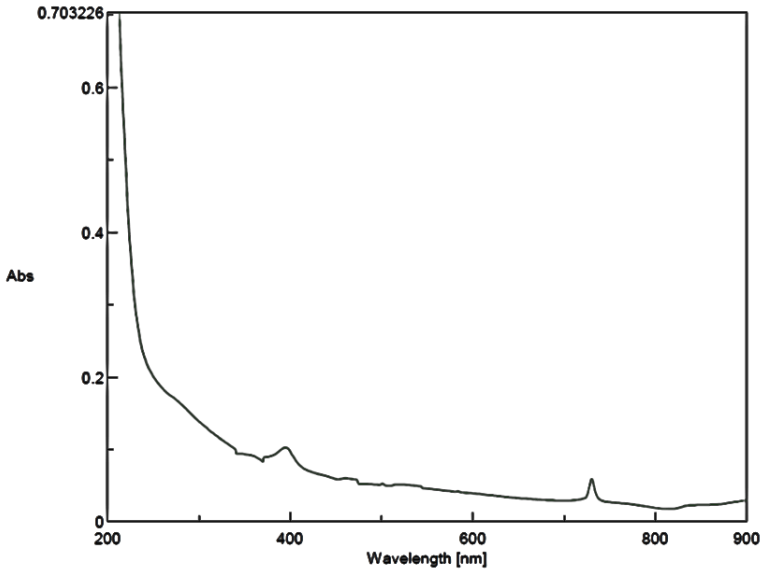

Figure 4 - Absorption spectrum of a 0.078 mg/L AuNP solution prepared in ASTM. The image was obtained through the Spectra Manager software. Abs = absorbance in optical density (OD) units.

Within this range, a significant and positive correlation between the absorbance and the AuNP was found for solutions in ultra-pure water (N = 24, r = 0.999, p = 0.000) and in ASTM (N = 24, r = 0.999, p = 0.000). The linear regression model fitted to ultra-pure water data had a Determination Coeficient (R) of 99.8% and is: AuNP concentration (mg/l) = 63.114 x absorbance value (OD units) + 0.267. The corresponding model fitted to ASTM data had a Determination Coefficient of 99.9% and is: AuNP concentration (mg/l) = 64.693 x absorbance value (OD units) + 0.21. The calibration curves with the fitted models are shown in Figure 5. No significant differences between the calibration curves in ultra-pure water and ASTM were found (ANCOVA, AuNP concentration: $F_{(1, 46)} = 28531.502$, p = 0.000; type of medium: $F_{(1, 46)} = 0.008$, p = 0.928), indicating no significant changes of size and shape of the AuNP tested in ASTM immediately after the preparation of the solutions. Thus, the spectrophotometry method is adequate to determine the concentrations of 5 nm AuNP particles in ASTM under the experimental conditions used.

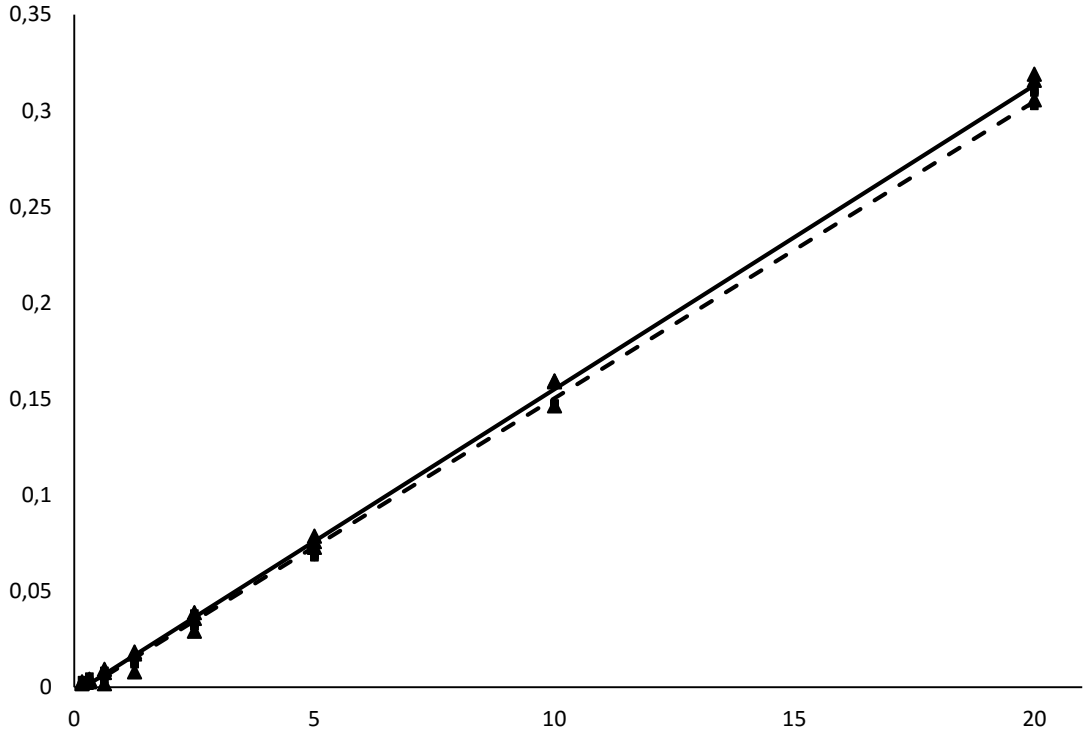

Figure 5 - AuNP calibration curves constructed by linear regression analysis of AuNP concentration vs absorbance at 520 nm. The solid line and the triangle markers refer to the model fitted to the solutions prepared in ultra-pure water. The dashed line and square markers refer to the model fitted to the solutions prepared in ASTM.

3.2.2 Behaviour of 5 nm AuNP Along 48 h

The potential changes of 5 nm AuNP in ASTM along 48 h was investigated comparatively to the changes in ultra-pure water solutions to select the time of test medium renewal during the toxicity tests with *D. magna*. A decrease in the absorbance value at 520 nm along time was observed for all AuNP concentrations (Table 7), with the highest variations occurring in the solutions prepared in ASTM. In ultra-pure water, the percentages of decay along 48 h ranged from 5.55% to 25.41% (Table 8). In ASTM (Table 8), the decay was higher ranging from 0.51% to 13.65% after 24 h, and from 7.18% to 55.97% after 48 h. These findings suggest a decay of 5 nm AuNP in the solutions, likely due to the formation of larger particles through aggregation.

At the 24 h time point, the highest percentage of degradation was found to be 18.77% for the 0.625 mg/L solution prepared in ultra-pure water. Most solutions prepared in ASTM had degradation values higher than 20% after 48 h, which indicates that it would not be appropriate to conduct the bioassays with medium renewal at each 48 h. The degradation values at 48 h are higher for the solutions prepared in ASTM for all concentrations tested, the only exception being the value for the 0.156 mg/L. This deviation could be explained by the fact that that concentration is at the lower limit of the linear regression model used to calculate the AuNP concentrations (refer to the section 3.2.1 for descriptions of the models).

The higher degradation observed in the solutions prepared in ASTM could be explained by the presence of several chemical substances that are present in ASTM but absent in ultra-pure water. The nutrients and vitamins present in ASTM could interact with the AuNP, causing particle degradation or promoting their aggregation, which would move the absorbance peak from 520 nm to a higher wavelength (Link and El-Sayed, 1999; Haiss *et al.*, 2007). The spectra at the different time points for the solutions prepared in ASTM also revealed that the absorbance peak shifts to the right with time, reaching 529 nm at the 48 h time point. This further supports the hypothesis that particle aggregation might have happened in the solutions prepared in ASTM.

The absorbance peak of AuNP shifts to longer wavelengths as particles size increases (Link and El-Sayed, 1999; Haiss *et al.*, 2007) and thus the absorbance peak at 520 nm decreases. According the OECD guidelines for toxicity testing with *D. magna*,

in both acute (OECD, 2004) and chronic (OECD, 2012) guidelines, the concentration of the tested substance should be maintained above 80% of the initial concentration along the exposure period, and thus the concentration decrease should not be higher than 20%. Because the decay of 5 nm AuNP in ASTM was higher than 20% at 48 h but not at 24 h (Table 8), during the bioassay with *D. magna* the test medium should be changed at least every 24 h to maintain the AuNP concentrations.

Table 7 - Absorbance values at 520 nm for the average of the three replicates prepared in ultra-pure water or ASTM, measured 24 h and 48 h after the preparation of the solutions. Values are the average of absorbance at 520 nm (3 replicate solutions) with the corresponding standard deviation within brackets. Abs = absorbance at 520 nm.

	Ultra-pure water							
	5 nm AuNP nominal concentrations (mg/l)							
Time (h)	0.156	0.312	0.625	1.25	2.5	5	10	20
0 h Abs	0.0004 (±0.0028)	0.0023 (±0.0013)	0.0070 (±0.0037)	0.0146 (±0.0050)	0.0347 (±0.0028)	0.0758 (±0.0074)	0.1550 (±0.0069)	0.3134 (±0.0087)
24 h Abs	0.0004 (±0.0013)	0.0023 (±0.0015)	0.0067 (±0.0041)	0.0138 (±0.0065)	0.0328 (±0.0082)	0.0721 (±0.0120)	0.1378 (±0.0210)	0.3084 (±0.0463)
48 h Abs	0.0003 (±0.0017)	0.0022 (±0.0011)	0.0059 (±0.0025)	0.0131 (±0.0033)	0.0310 (±0.0132)	0.0700 (±0.0154)	0.1320 (±0.0204)	0.2709 (±0.0566)

	ASTM							
	5 nm AuNP nominal concentrations (mg/l)							
Time (h)	0.156	0.312	0.625	1.25	2.5	5	10	20
0 h Abs	0.0014 (±0.0020)	0.0031 (±0.0031)	0.0067 (±0.0042)	0.0135 (±0.0061)	0.0341 (±0.0115)	0.0688 (±0.0179)	0.1473 (±0.0260)	0.3074 (±0.0873)
24 h Abs	0.0012 (±0.0013)	0.0030 (±0.0033)	0.0063 (±0.0042)	0.0134 (±0.0069)	0.0314 (±0.0098)	0.0666 (±0.0112)	0.1466 (±0.0231)	0.3051 (±0.0380)
48 h Abs	0.0011 (±0.0014)	0.0029 (±0.0017)	0.0055 (±0.0032)	0.0101 (±0.0053)	0.0241 (±0.0083)	0.0504 (±0.0106)	0.1020 (±0.0462)	0.1354 (±0.0497)

Table 8 – Decay of 5 nm AuNP (%) in solutions prepared in ultra-pure water or ASTM, at 24 h and 48 h. The decay was calculated as: Decay (%) = 100 - (abs2 x 100/abs1). Abs1 = absorbance at 520 nm at 0h. Abs2 = absorbance at 520 nm at 24 or 48h.

	Ultra-pure water							
	5 nm AuNP nominal concentrations (mg/l)							
Time (h)	0.156	0.312	0.625	1.25	2.5	5	10	20
24 h decay (%)	0.54	0.13	18.77	5.36	5.42	4.80	11.10	1.61
48 h decay (%)	25.41	5.55	16.10	10.09	10.61	7.65	14.84	13.57

	ASTM							
	5 nm AuNP nominal concentrations (mg/l)							
Time (h)	0.156	0.312	0.625	1.25	2.5	5	10	20
24 h decay (%)	13.21	3.06	4.45	0.53	13.65	3.21	0.51	0.75
48 h decay (%)	19.24	7.18	18.25	25.16	29.30	26.74	30.62	55.97

3.3 MP Calibration Curves

For the solutions with MP concentrations below or equal to 0.047 mg/L, the fluorescence intensity (FI) values obtained became very close to zero or had negative values, indicating no sensitivity of the method in that range. Because of this, the calibration curves were made with data from the solutions with concentrations of 0.094, 0.187, 0.375, 0.75, 1.5, 3, and 6 mg/L.

Within this range, a significant and positive correlation between the fluorescence intensity and the MP was found for solutions in ultra-pure water (N = 24, r = 0.998, p = 0.000) and in ASTM (N = 24, r = 0.997, p = 0.000). The linear regression model fitted to ultra-pure water data had a Determination Coefficient (R) of 99.6% and is: MP concentration (mg/l) = 0.023 x fluorescence value (FI units) + 0.027. The corresponding

model fitted to ASTM data had a Determination Coefficient of 98.1% and is: MP concentration (mg/l) = 0.016 x fluorescence value (FI units) + 0.217. The calibration curves with the fitted models are shown in Figure 6. No significant differences between the calibration curves in ultra-pure water and ASTM were found (ANCOVA, MP concentration: $F_{(1, 46)} = 1023.699$, $p = 0.000$; type of medium: $F_{(1, 46)} = 0.337$, $p = 0.564$), indicating no significant changes of size and shape of the MP tested in ASTM immediately after the preparation of the solutions. Thus, the spectrofluorimetry method is adequate to determine the concentrations of 5 µm MP particles in ASTM under the experimental conditions used.

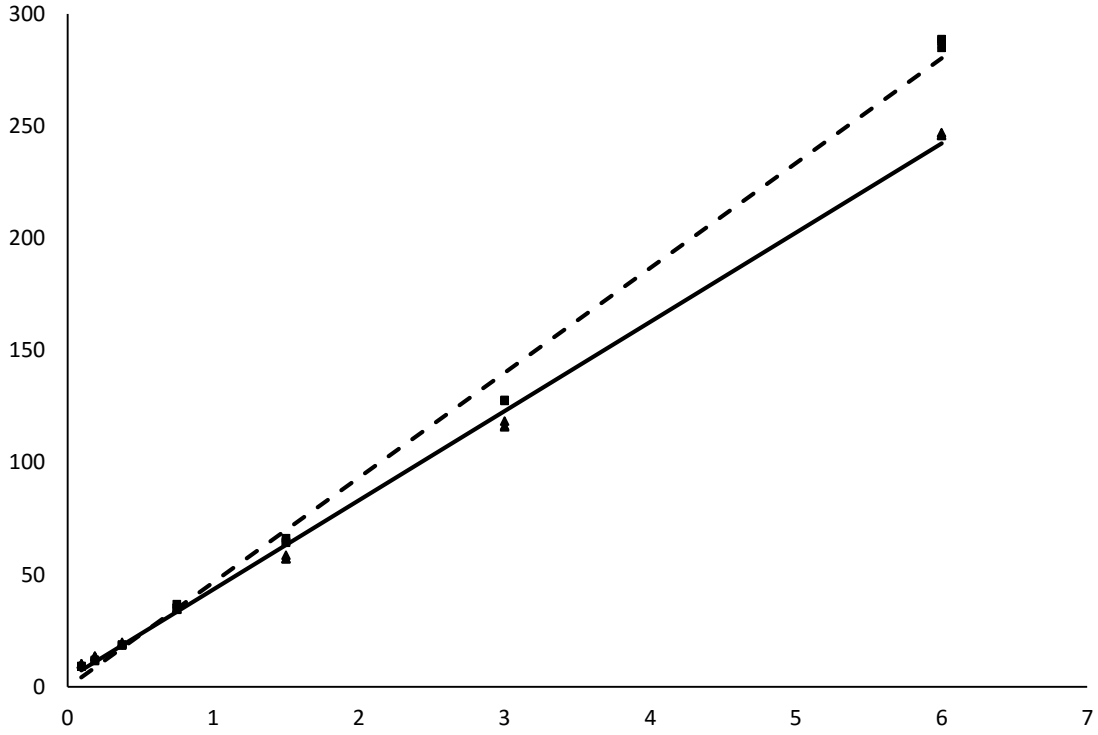

Figure 6 - MP calibration curves constructed by linear regression analysis of MP concentration vs fluorescence. The solid line and the triangle markers refer to the model fitted to the solutions prepared in ultra-pure water. The dashed line and square markers refer to the model fitted to the solutions prepared in ASTM.

3.3.1 Behaviour of 5 µm MP Along 48 h

The potential changes of 5 µm MP in ASTM along 48 h was investigated comparatively to the changes in ultra-pure water solutions to select the time of test medium renewal during the toxicity tests with *D. magna*. A decrease in the fluorescence along with time was observed for all MP concentrations (Table 9).

In ultra-pure water, the percentages of decay along 48h ranged from 27.60% to 49.53% (Table 10). In ASTM (Table 10), the decay was higher ranging from 26.07% to 67.02% after 48h. It was also observed that the plastic particles sedimented in the bottom of the test beakers as soon as 24 hours after the preparation of the solutions. During the bioassays, this would pose a problem, as it would not be possible to homogenize the test solutions without disturbing the test animals. In order to maintain a stable concentration of MP, it would be necessary to increase the frequency of media renewal during the assay, which would highly increase the amount of labor necessary to conduct the assay.

Table 9 - Fluorescence values at 575 nm for the excitation wavelength and 607 nm for the emission wavelength for the average of the three replicates prepared in ultra-pure water or ASTM, measured 24 and 48h after the preparation of the solutions. Values are the average of fluorescence (3 replicate solutions) with the corresponding standard deviation within brackets. Fl = fluorescence at 575 nm for the excitation wavelength and 607 nm for the emission wavelength.

	Ultra-pure water						
Time	5 µm MP nominal concentrations (mg/l)						
(h)	0.094	0.187	0.375	0.75	1.5	3	6
0 h Fl	10.2431 (±1.3745)	13.5630 (±0.3742)	19.3937 (±1.5665)	34.6903 (±0.4002)	57.6002 (±0.7178)	116.8704 (±2.8861)	246.2501 (±5.7552)
24 h Fl	7.4593 (±1.2621)	8.8638 (±1.6312)	11.8474 (±1.0866)	30.2271 (±3.5547)	48.6767 (±6.7721)	105.4312 (±12.2924)	238.4524 (±24.2731)
48 h Fl	7.4160 (±0.4879)	8.5692 (±1.5545)	9.787 (±1.3871)	23.2301 (±3.0924)	34.6980 (±5.8035)	72.3411 (±10.8814)	173.2843 (±21.2395)

	ASTM						
Time	5 µm MP nominal concentrations (mg/l)						
(h)	0.094	0.187	0.375	0.75	1.5	3	6
0 h Fl	9.1210 (±0.3618)	11.6705 (±0.1847)	18.6438 (±0.8094)	35.7214 (±1.1417)	65.0242 (±2.8602)	127.6271 (±8.3282)	287.1753 (±23.8561)
24 h Fl	6.4514 (±0.3784)	7.4524 (±3.5822)	12.4902 (±2.0454)	24.7928 (±8.1922)	42.7572 (1±0.6275)	98.4501 (±15.2556)	218.7637 (±20.4981)
48 h Fl	6.7417 (±0.5301)	7.9506 (±2.0765)	9.4903 (±3.4378)	11.7817 (±5.0577)	25.7604 (±6.1738)	74.4543 (±14.1071)	156.3041 (±23.1085)

Table 10 – Decay of 5 µm MP (%) in solutions prepared in ultra-pure water or ASTM, at 24h and 48h. The decay was calculated as: Decay (%) = 100 - (Fl2 x 100/Fl1). Fl1 = fluorescence (575 nm for the excitation wavelength and 607 nm for the emission wavelength) at 0h. Fl2 = fluorescence (575 nm for the excitation wavelength and 607 nm for the emission wavelength) at 24 h or 48h.

	Ultra-pure water						
	5 µm MP nominal concentrations (mg/l)						
Time (h)	0.094	0.187	0.375	0.75	1.5	3	6
24 h decay (%)	27.18	34.65	38.91	12.87	15.49	9.79	3.17
48 h decay (%)	27.60	36.82	49.53	33.04	39.76	38.10	29.63

	ASTM						
	5 µm MP nominal concentrations (mg/l)						
Time (h)	0.094	0.187	0.375	0.75	1.5	3	6
24 h decay (%)	29.26	36.16	33.01	30.62	34.26	22.86	23.82
48 h decay (%)	26.07	31.88	49.11	67.02	60.39	41.66	45.57

3.4. Chronic Assay with AuNP and MP

3.4.1. General Conditions, and Actual Concentrations and Decay of AuNP and MP in Test Media

Throughout the duration of the assay (21 days), for each test beaker, the variation of the pH of the testing media was less than 1 unit, the variation of the temperature was less than 1ºC and the dissolved oxygen remained above 3 mg/L at all times.

During the assay, AuNP and MP concentrations were monitored at beginning of the exposure period and then at each 24h, at the time of test media renewal, in both fresh and old media. The values of the absorbance of the freshly prepared media, the media

prepared in the previous day and the AuNP, the deviation of actual concentrations from nominal ones, and the decay of AuNP and MP in test media are shown in Table 11 and Table 12, respectively. The deviation of actual AuNP concentrations relatively to nominal ones, ranged from 0.1437% (±0.2486) to 18.1228% (±0.0026). Because such deviations were lower than 20%, the actual concentrations will be expressed as the nominal ones (OECD, 2012). Corresponding deviations for MP ranged from 7.4286% (±0.5102) to 8.4072% (±2.1574), thus actual MP concentrations will be also expressed as the nominal ones. The highest AuNP decay was 7.4%, thus the concentrations of 5 nm AuNP were maintained above 80% during the bioassay. MP decay always exceeded 20%, probably due to the sedimentation of the particles. The fluorescence readings of the test solutions containing 0.02 mg/L of MP could not be read due to the lack of sensitivity of the method regarding concentrations of this magnitude.

Table 11 - Absorbance at 520 nm of the different test solutions from the chronic assay. The AuNP actual concentrations were estimated using the linear regression model fitted to ultra-pure water presented in section 3.2.1 Values are presented as the average of 2 to 10 replicates with corresponding standard deviation. Only the values referring to the treatments containing AuNP are presented. Abs = absorbance value at 520 nm in optical density (OD) units. Old media = media prepared in the previous day. Fresh media = freshly prepared media. The decay was calculated as: Decay (%) = 100 - (abs_{Old} x 100/abs_{Fresh}). Abs_{Fresh} = absorbance at 520 nm of the fresh media. Abs_{Old} = absorbance at 520 nm of the old media. The deviation from the actual concentration to the nominal ones was calculated as: Deviation (%) = 100 - ($Actual_{Conc}$ x 100/$Nominal_{Conc}$). $Actual_{Conc}$ = concentration estimated using the linear regression model. $Nominal_{Conc}$ = nominal concentration of AuNP. Conc. = concentration.

Test Solution	Nominal AuNP conc.(mg/L)	abs of fresh media	AuNP Actual conc. (mg/l)	Deviation of actual conc. to nominal ones (%)	abs of old media	Decay (%)
2 mg/L AuNP	2.00	0.0287 (±0.0037)	2.0224 (±0.4445)	1.1186 (±0.0041)	0.0267 (±0.0078)	6.7
0.2 mg/L AuNP	0.2	0.0004 (±0.0003)	0.2362 (±0.2299)	18.1228 (±0.0017)	0.0004 (±0.0002)	0.15
0.2 mg/L AuNP + 0.02 mg/L MP	0.20	0.0004 (±0.0002)	0.2362 (±0.2236)	18.1228 (±0.0026)	0.0004 (±0.0001)	0.26
0.2 mg/L AuNP + 0.2 mg/L MP	0.20	0.0003 (±0.0002)	0.2299 (±0.2236)	14.9671 (±0.0125)	0.0003 (±0.0025)	0.18
2 mg/L AuNP + 0.02 mg/L MP	2.00	0.0283 (±0.0068)	1.9971 (±0.6402)	0.1437 (±0.2486)	0.0266 (±0.0034)	5.8
2 mg/L AuNP + 0.2 mg/L MP	2.00	0.0289 (±0.0057)	2.0350 (±0.5707)	1.7497 (±0.1572)	0.0267 (±0.0066)	7.4

Table 12 – Fluorescence intensity (FI) values at 575 nm for the excitation wavelength and 607 nm for the emission wavelength, of the different test solutions from the chronic assay. The MP actual concentrations were estimated using the linear regression model fitted to ultra-pure water presented in section 3.3.1 Values are presented as the average of 2 to 10 replicates with corresponding standard deviation. Only the values referring to the treatments containing MP are presented. Fluorescence = fluorescence at 575 nm for the excitation wavelength and 607 for the emission wavelength in FI units. Old media = media prepared in the previous day. Fresh media = freshly prepared media. The decay was calculated as: Decay (%) = 100 - (Fl_{Old} x 100/Fl_{Fresh}). Fl_{Fresh} = fluorescence (at 575 nm for the excitation wavelength and 607 for the emission wavelength) of the fresh media. Fl_{Old} =fluorescence (at 575 nm for the excitation wavelength and 607 for the emission wavelength) of the old media. The deviation from the actual concentration to the nominal ones was calculated as: Deviation (%) = 100 - ($Actual_{Conc}$ x 100/$Nominal_{Conc}$). $Actual_{Conc}$ = concentration estimated using the linear regression model. $Nominal_{Conc}$ = nominal concentration of MP. Conc. = concentration. NA = Not applicable

Test solution	Nominal MP conc. (mg/L)	Fluorescence of fresh media	MP Actual concentration (mg/l)	Deviation of actual conc. to nominal ones (%)	Fluorescence of old media	Decay (%)
0.02 mg/L MP	0.02	NA	NA	NA	NA	NA
0.2 mg/L MP	0.20	8.2528 (±2.6011)	0.2168 (±0.0868)	8.4072 (±2.1574)	6.5207 (±2.1548)	36,4
0.2 mg/L AuNP + 0.02 mg/L MP	0.02	NA	NA	NA	NA	NA
0.2 mg/L AuNP + 0.2 mg/L MP	0.20	8.1677 (±2.3577)	0.2149 (±0.0812)	7.4286 (±0.5102)	8.3911 (±0.2184)	28.14
2 mg/L AuNP + 0.02 mg/L MP	0.02	NA	NA	NA	NA	NA
2 mg/L AuNP + 0.2 mg/L MP	0.20	8.1850 (±1.5856)	0.2153 (±0.0635)	7.6275 (±3.5148)	7.6617 (±2.1548)	31.5

3.6.2 Mortality

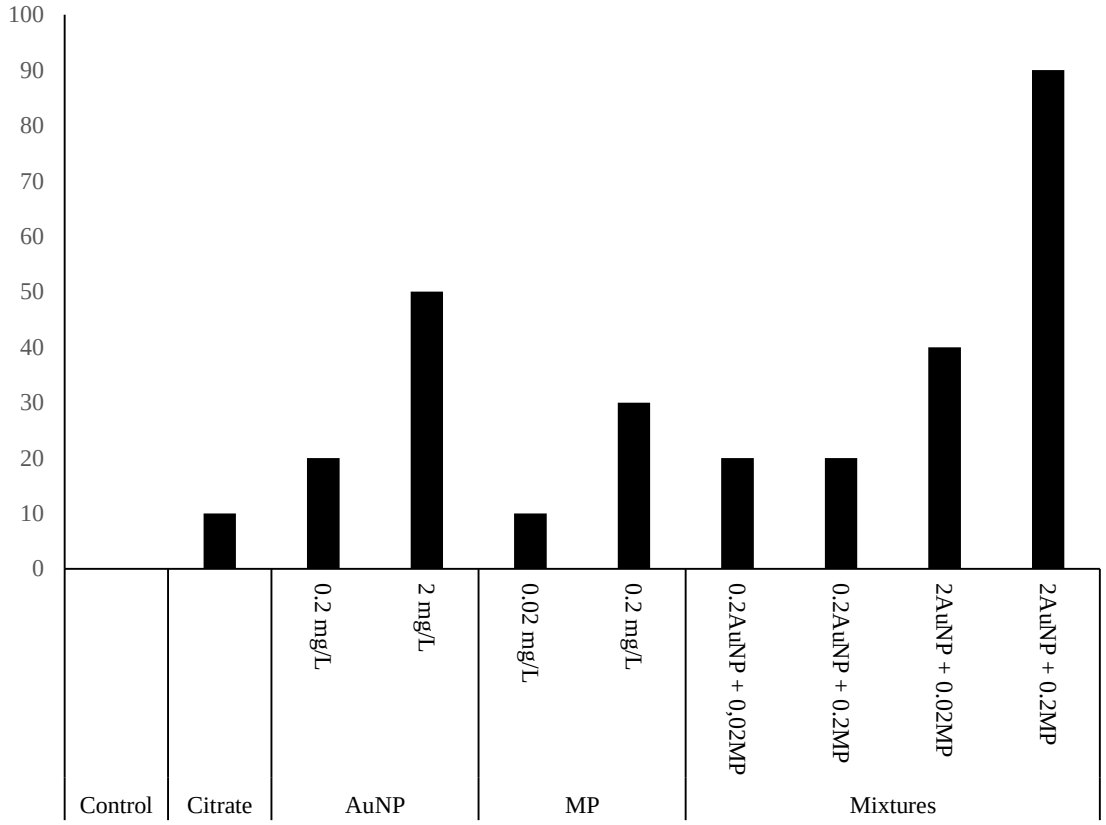

Figure 7 – Total percentage of mortality recorded in parental animals over the 21-day exposure period per treatment. 0.2AuNP+0.02MP = mixture of 0.2 mg/L AuNP + 0.02 mg/L MP. 0.2AuNP+0.2MP = mixture of 0.2 mg/L AuNP + 0.2 mg/L MP. 2AuNP+0.02MP = mixture of 2 mg/L AuNP + 0.02 mg/L MP. 2AuNP+0.2MP = mixture of 2 mg/L AuNP + 0.2 mg/L MP.

There was no parental mortality in the control group, while all the other treatments induced some degree of parental mortality. The percentages of the total mortality recorded during the exposure period per treatment are shown in Figure 7. The mortality induced by AuNP, when tested alone, was 20% and 50% at the lowest and highest concentrations tested, respectively. In single exposures to MP, the mortality was slightly lower being 10% and 30% at the lowest and highest concentrations tested. The sum of the percentages of mortality recorded at the highest concentrations of AuNP and MP tested in single exposures was 80%, while the mortality induced by the mixture treatment containing similar concentrations of the two substances was 90%. Because the effect

induced by the mixture is higher than the sum of the effects induced by the two agents when tested alone, the type of toxicological interaction evaluated through mortality data seems to be synergistic, as indicated in Klaassen (2013).

Because there was only one animal alive at the end of the assay in the mixture of 2mg/L AuNP + 0.2 mg/L MP, the data regarding this treatment should not be included in the statistical analysis of the reproduction parameters and somatic growth.

3.4.2. Effects on Reproduction

The data relative to reproduction parameters are shown in Figures 8 and 9. Significant differences among treatments were found for the average of viable juveniles produced per female (1-ANOVA, $F_{(8, 59)} = 116.824$, $p = 0.000$), the average of immobile juveniles produced per female (1-ANOVA, $F_{(8, 59)} = 13.315$, $p = 0.000$), the average of aborted eggs produced per female (1-ANOVA, $F_{(8, 59)} = 106.165$, $p = 0.000$), average number of broods produced per female (1-ANOVA, $F_{(8, 59)} = 31.225$, $p = 0.000$) and for the day of release of the first brood (1-ANOVA, $F_{(8, 59)} = 14.114$, $p = 0.000$). Significant differences between the control group and the citrate-control group were found for two of the reproductive parameters analyzed. Therefore, at the concentration tested, citrate by itself induces adverse effects on *D. magna* reproduction. Since all the treatments containing AuNP also contain citrate, especial care to this aspect should be taken during the interpretation of the results of bioassays aimed at testing the toxicity of nanoparticles stabilized in citrate solutions to *D. magna*.

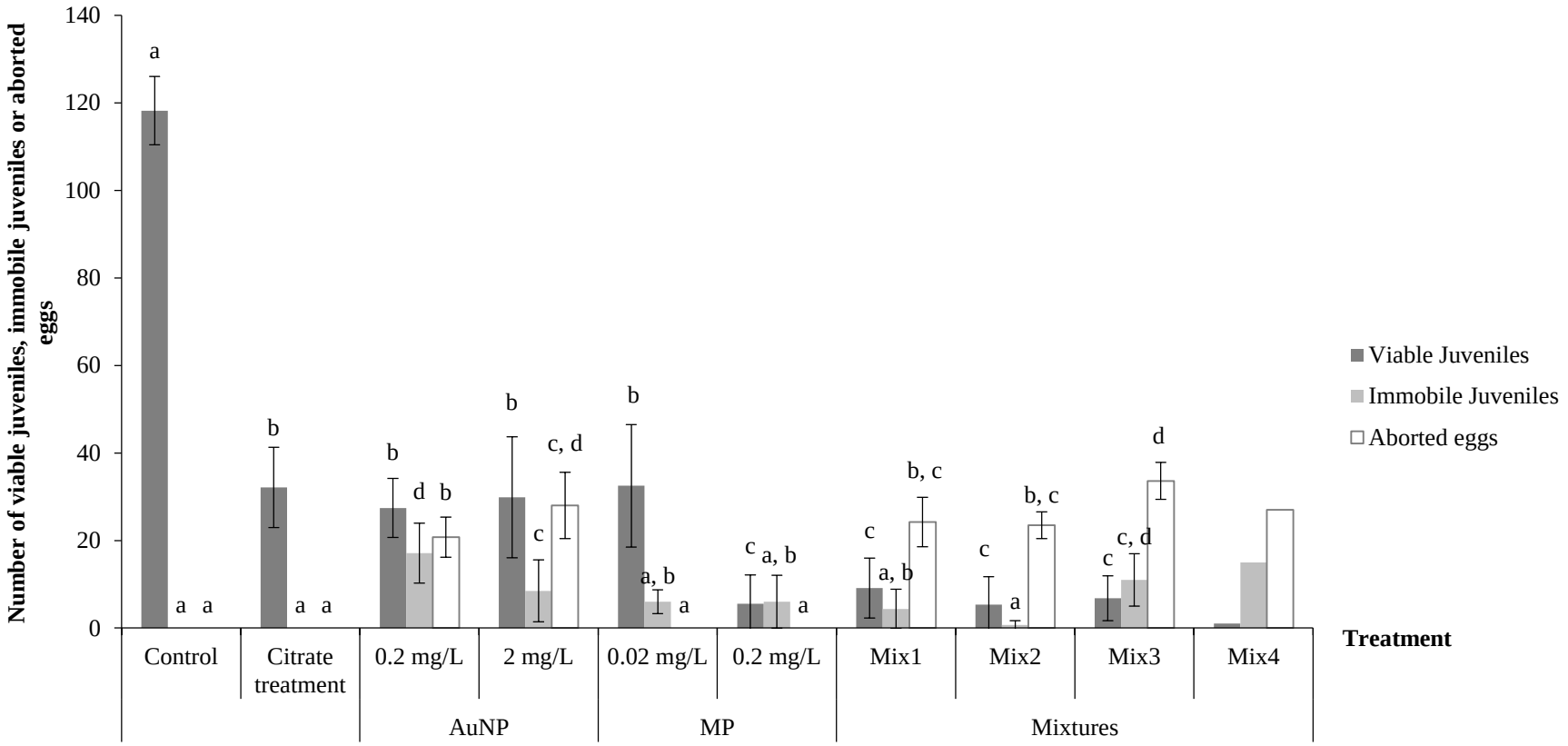

Figure 8 – Average and standard deviation of the total number of viable juveniles, immobile juveniles and aborted eggs produced per parental animal, per treatment, at the end of the assay. The lowercase letters above the error bars indicate treatments whose effects were not statistically different among themselves. Mix1 = mixture of 0.2 mg/L AuNP + 0.02 mg/L MP. Mix2 = mixture of 0.2 mg/L AuNP + 0.2 mg/L MP. Mix3 = mixture of 2 mg/L AuNP + 0.02 mg/L MP. Mix4 = mixture of 2 mg/L AuNP + 0.2 mg/L MP.

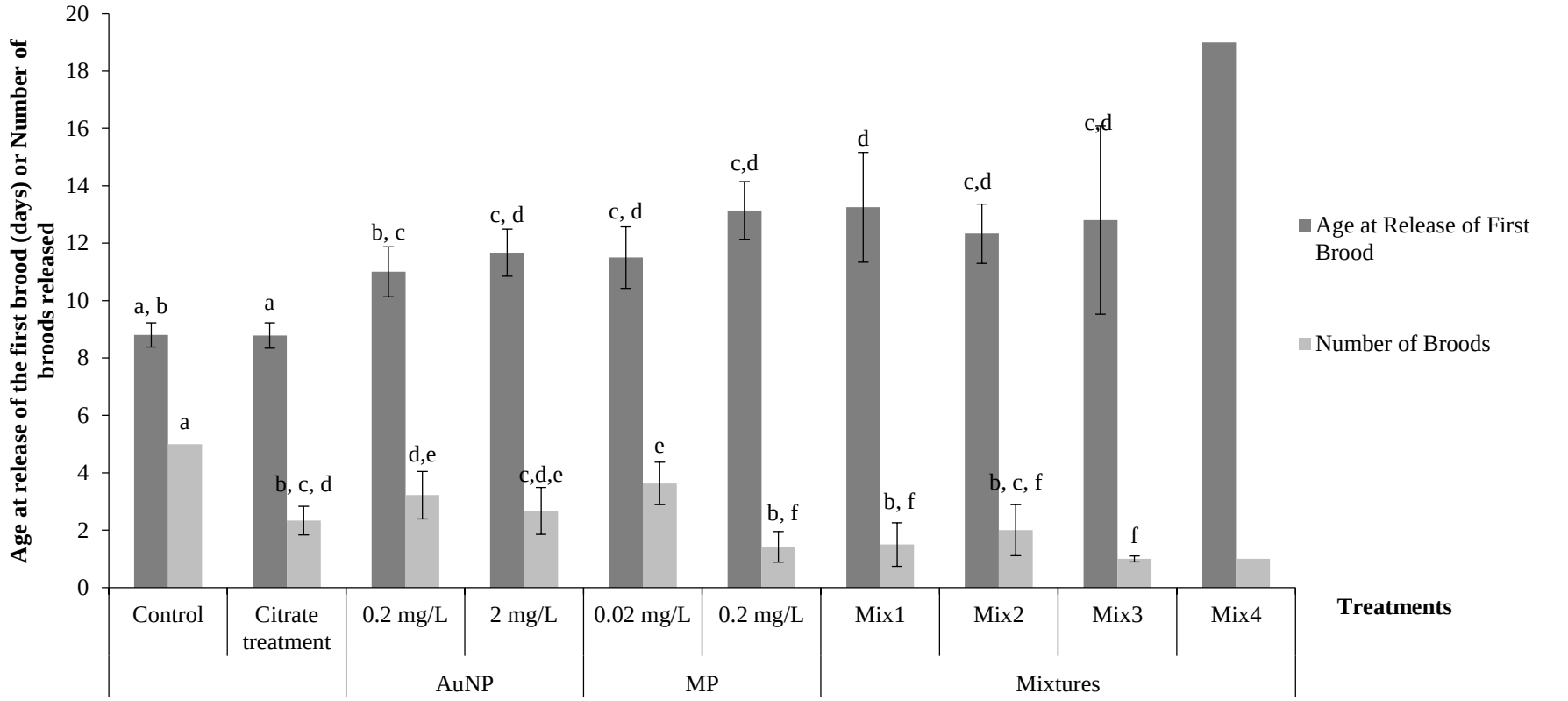

Figure 9 - Average and standard deviation of the age (in days) of the release of the first brood and total number of broods produced per parental animal, per treatment, at the end of the assay. The lowercase letters above the error bars indicate treatments whose effects not statistically different among themselves. Mix1 = mixture of 0.2 mg/L AuNP + 0.02 mg/L MP. Mix2 = mixture of 0.2 mg/L AuNP + 0.2 mg/L MP. Mix3 = mixture of 2 mg/L AuNP + 0.02 mg/L MP. Mix4 = mixture of 2 mg/L AuNP + 0.2 mg/L

The animals exposed to the AuNP alone treatments produced a lower number of viable juveniles compared with the control group. However, the effect on viable juveniles of the AuNP treatments was not statistically different from the effects caused by the citrate treatment, which means that the effects observed might be attributable to the citrate buffer, not the AuNP. The animals in the control group and in the citrate treatment produced no immobile juveniles. The only treatments that produced a significantly increased number of immobile juveniles were the ones where AuNP were present. The animals exposed to the lower concentration of AuNP alone produced more immobile juveniles than the animals exposed to the higher AuNP alone concentration. This might be a result of the hormesis effect, a phenomenon in which a low dose of a toxic agent causes a stimulation of a biological parameter, while higher doses cause inhibition of such parameter. Hormesis is thought to happen as a compensatory mechanism after disruption of homeostasis (Jemec *et al.*, 2007). The increase in the production of immobile juveniles could result from an evolutionary strategy developed by *D. magna*, in which an increase in fecundity could lead to a higher number of total juveniles produced, thus increasing the probability of some of those juveniles surviving. The AuNP alone treatments also caused an increase in the number of aborted eggs, with the highest concentration of AuNP causing higher numbers of aborted eggs. All treatments caused a decrease in the number of broods, and the release of the first brood was delayed in the animals exposed to the treatment with the higher AuNP alone concentration. However, the effects on the number of broods produced per female induced by the citrate treatment were similar to the effects induced by the AuNP alone treatments, which means that the observed effects might be attributable to the citrate buffer, not the AuNP.

These results suggest that AuNP, individually, did affect the reproduction potential of *D. magna*, causing a delay in the release of the first brood and increasing the amount of immobile juveniles and aborted eggs produced per female. Direct exposure to AuNP could cause a variety of detrimental effects on the daphnids. The increase of ROS production is a known effect that AuNP can induce in living organisms (Xia *et al*., 2006; Auffan *et al.*, 2008; Gou *et al.*, 2010) and it has already been observed in aquatic organisms. 40 nm AuNP caused an increase in the activity of catalase and superoxide dismutase, which play an important role in the defense against oxidative stress, in the bivalve mollusk *Scrobicularia plana* (Pan *et al.*, 2012). Oxidative stress was also observed in *Mytilus edulis* after exposure to 5 nm AuNP (Tedesco *et al.*, 2010). There

are also other types of detrimental effects that AuNP could have on *D. magna*, from genotoxicity (Gou *et al.,* 2010), which could affect the development of the embryos, resulting in the observed increase in aborted eggs and immobile juveniles, to the disruption of subcellular organelles (Foley *et al.,* 2002; Williams *et al.,* 2009; Sengstock *et al.,* 2011; Elsaesser and Howard, 2012). It is also possible that AuNP cause direct effects on the eggs and embryos, since the contaminated water can enter the brood pouch. However, the nature of such effects was impossible to determine during the present work. These effects could also be due to a decrease in food quality, modifications in the allocation of resources or a combination of both. AuNP adsorption to green microalgae has already been reported. For example, Renault et al reported that AuNP adsorb into the green alga *Scenedesmus subspicatus*, causing algae mortality after 24 h of exposure (Renault *et al.*, 2008). It's possible that the AuNP adsorbed to the *C. vulgaris* cells that were used to feed the daphnids during the assay. This could cause a decrease in the quality of the algae as a food source, which would then affect the daphnids' reproductive fitness. The microalgae could also have been a vector for AuNP ingestion by the daphnids, increasing the total amount of AuNP ingested.

The animals exposed to the MP alone treatments produced fewer viable juveniles compared with the control group, with the higher MP concentration causing a significant decrease compared with the lower MP concentration. The animals exposed to the MP alone treatments produced more immobile juveniles compared with the control, even though the difference was not found to be statistically significant. MP also affected the age of release of the first brood and the total number of broods produced per female, with the highest MP concentration resulting in a lower number of broods, compared with the lower MP concentration. The number of aborted eggs produced per female was not affected by MP, compared with the control group.

MP seem to interfere with *D. magna* reproduction, decreasing the amount of viable juveniles, delaying the release of the first brood and reducing the total number of broods released. The effects that MP might cause on *D. magna* include the blockage of the intestinal tract, damage through internal abrasion or interference with the filtering apparatus (Eerkes-Medrano *et al.*, 2015). It's also possible that the daphnids ingest the MP instead of their actual food source, causing a false sense of satiation which can lead to starvation or impairment of several biological processes due to the decrease in caloric

intake (Cole *et al*., 2011). A reduction of feeding activity due to the presence of MP has been reported in *Arenicola marina* (Wright *et al.*, 2013a) and *Eugerres brasilianus* (Ramos *et al.*, 2012) after exposure to MP. The accumulation of MP in the gut of *D. magna* has already been reported as well (Rosenkranz *et al.*, 2009), although it's uncertain whether it caused a decrease in caloric intake or not.

The animals exposed to the mixtures containing the lower AuNP concentrations (0.2 mg/L AuNP mixed with 0.02 mg/L MP or 0.2 mg/L MP) produced a lower number of viable juveniles, compared with their correspondent AuNP alone treatments. The animals exposed to the mixture of 2 mg/L AuNP + 0.02 mg/L MP also had a lower number of viable juveniles when compared with the animals exposed to the treatment with the highest concentration of AuNP alone and with the treatment with the lowest concentration of MP alone. As for the number of immobile juveniles produced per female, the effects caused by the mixtures containing the lower AuNP concentrations (0.2 mg/L AuNP mixed with 0.02 mg/L MP or 0.2 mg/L MP) were not significantly different from each other nor from their correspondent MP alone treatments. They were different from the lowest concentration of AuNP alone, which suggests that the presence of MP might have contributed to the increase in the number of immobile juveniles, even though the concentration of MP seems to be irrelevant, as the animals exposed to the mixtures containing the lower AuNP concentrations (0.2 mg/L AuNP mixed with 0.02 mg/L MP or 0.2 mg/L MP) had the same amount of immobile juveniles despite having different concentrations of MP. This hypothesis is further supported by the fact that the effects caused by the different MP alone treatments did not differ among themselves. The animals in the mixtures showed a similar production of aborted eggs when compared with their correspondent AuNP alone treatments, which suggests that MP did not interfere with the number of aborted eggs while mixed with AuNP. Regarding the total number of broods produced per female, the presence of MP, even in the lower concentration, seems to interfere with the effects of AuNP in the mixtures. The animals exposed to the mixtures containing the lower AuNP concentrations (0.2 mg/L AuNP mixed with 0.02 mg/L MP or 0.2 mg/L MP) had a lower number of broods when compared with the animals exposed to the correspondent AuNP alone treatments and were similar to the animals exposed to the highest MP alone concentration. The animals exposed to the mixture of 2 mg/L AuNP + 0.02 mg/L MP also had a lower number of broods when compared with the animals exposed to the treatment with the higher AuNP alone concentration. However, the age at

the release of the first brood doesn't seem to be affected by the interaction between AuNP and MP. With the exception of the mixture of 0.2 mg/L AuNP + 0.02 mg/L MP, the mixtures did not deviate from the correspondent AuNP or MP alone treatments.

3.4.3. Effects on Somatic Growth

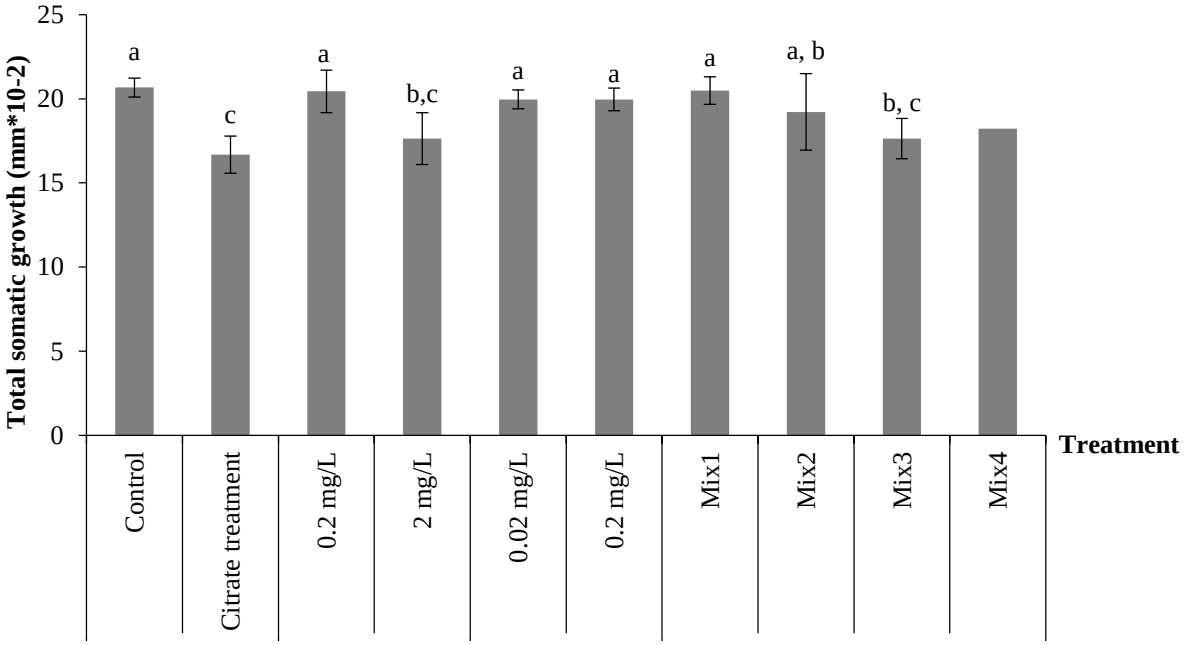

Figure 10 - Average and standard deviation of the total somatic growth per parental animal, per treatment, at the end of the assay. The lowercase letters above the error bars indicate treatments whose effects not statistically different among themselves. Mix1 = mixture of 0.2 mg/L AuNP + 0.02 mg/L MP. Mix2 = mixture of 0.2 mg/L AuNP + 0.2 mg/L MP. Mix3 = mixture of 2 mg/L AuNP + 0.02 mg/L MP. Mix4 = mixture of 2 mg/L AuNP + 0.2 mg/L.

The data relating to the average of total somatic growth per female and per treatment at the end of the assay are presented in Figure 10. The citrate treatment caused a decrease in the total somatic growth similar to the effects of the treatment with the higher AuNP alone concentration and to the mixture of 2 mg/L AuNP + 0.02 mg/L MP. All other treatments were similar to the control group. This suggests that the presence of high quantities of citrate causes a decrease in total growth while MP does not, either alone or in mixture.

3.4.4. AuNP and MP Interactions and General Discussion

The information about the effects of AuNP on the number of viable juveniles produced per female, total number of broods released and total somatic growth is inconclusive, as the citrate treatment caused similar effects to those observed in the animals exposed to the different AuNP treatments. For the remaining criteria, a summary of the statistical parameters obtained in the 2-ANOVA test are presented in Table 13.

Table 13 – Summary of the statistical parameters obtained in the two-way analysis of variance (2-ANOVA). Only the biological parameters that didn't show differences between the control and citrate treatment after the one-way ANOVA (1-ANOVA) analysis are presented. df = degrees of freedom. AuNP = Gold nanoparticles concentration. MP = Microplastics concentration.

	Sum of Squares	df	Mean Square	F value	p
Immobile Juveniles					
AuNP	204.292	2	102.146	4.387	0.017
MP	143.026	2	71.513	3.071	0.055
AuNP X MP	1278.585	3	426.195	18.302	0.000
Aborted eggs					
AuNP	9344.575	2	4672.287	300.202	0.000
MP	102.818	2	51.409	3.303	0.045
AuNP X MP	60.960	3	20.320	1.306	0.283
Age at first brood released					
AuNP	22.973	2	11.486	5.892	0.005
MP	71.060	2	35.530	18.225	0.000
AuNP X MP	22.855	3	7.618	3.908	0.014

The 2-ANOVA analysis revealed that interactions between AuNP and MP were significant when looking at the age of release at the first brood and immobile juveniles, but not for the number of aborted eggs produced per female. These results show that AuNP and MP interact with each other, causing an intensification of the effects observed

in the AuNP or MP treatments containing the isolated substances. One of the potential sources of MP toxicity lies on their ability to adsorb other pollutants present on the contaminated waters. It is possible that the AuNP absorbed to the MP, which then acted as a vector for AuNP delivery to the daphnids. This would exacerbate the toxic effects associated with AuNP, if such effects were dependent on the quantity of AuNP ingested by the animals. Should either the MP or AuNP individually induce toxic effects on *D. magna*, the debilitated animals would become more vulnerable to any effect caused by the presence of the second pollutant. For example, if the AuNP caused oxidative stress in *D. magna*, the resources needed to activate anti-oxidative defense mechanisms could become compromised by a decrease in food quality potentially caused by the presence of MP.

4. Conclusions and Future Prospects

The results obtained throughout this work allowed for the rejection of all three null hypothesis tested. The first hypothesis, that AuNP were not toxic to *D. magna* at water concentrations in the ppm range was rejected, as the concentrations of AuNP tested (0.2 mg/l and 2 mg/L) caused an increased number of immobile juveniles and aborted eggs produced per female, delayed the release of the first brood and caused parental mortality. The hypothesis that MP were not toxic to *D. magna* at water concentrations in the ppm range was also rejected, as the concentrations of these particles tested (0.02 mg/l and 0.2 mg/l) also caused parental mortality, decreased the number of viable juveniles produced per female, delayed the release of the first brood and decreased the total number of broods released. Finally, the third null hypothesis was rejected as well, as the presence of MP in mixture with AuNP delayed the release of the first brood and increased the number of immobile juveniles produced per female, in relation to the effect caused by the substances when tested separately.

Furthermore, significant interactions between MP and citrate buffer were also found, affecting the number of viable juveniles produced per female and the total number of broods released. While the study of the interactions between MP and the citrate buffer were not included in the aims of this work, these findings further illustrate the need to study not only each pollutant individually, but also their interactions when mixed together.

The presence of MP in aquatic ecosystems is already well established, with no signs of reverting in the foreseeable future (Eerkes-Medrano *et al.*, 2015). Simultaneously, the production of AuNP is likely to increase as their range of applications widens (Dreaden *et al.*, 2012). This raises concerns about the potential toxicity associated with these particles, both by the general population as well as by the regulatory entities. Future works should focus on determining the EC_{50}, LOEC, NOEC and other parameters of toxicity induced by these particles, in order to facilitate the regulation of their usage, disposal and to allow the monitoring of water quality. There is still much work to do until a proper risk assessment of these particles is concluded. It would also be important to uncover the source of AuNP and MP induced toxicity. While many hypothesis exist, more knowledge is needed in order to truly understand how and why these particles affect

freshwater organisms. It is also imperative to conduct toxicity studies of AuNP and MP exposure at the different trophic levels, as the particles' toxicity could change significantly throughout the food web. Bioaccumulation and biotransformation are among the phenomena that could affect the behavior and consequent toxicity of AuNP and MP.

5. References

Amendola, V. and Moreno M. 2009. Size Evaluation of Gold Nanoparticles by UV-vis Spectroscopy. *The Journal of Physical Chemistry C* **11**: 4277–4285.

Andrady, A. L. 2011. Microplastics in the marine environment. *Marine Pollution Bulletin* **62**(8): 1596–1605.

Andrady, A. and Neal, M. 2009. Applications and societal benefits of plastics. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**: 1977–1984.

American Society for Testing and Materials (ASTM), 1980. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians, Report E-729-80.

American Society for Testing and Materials (ASTM), PhiladelphiaAmerican Society for Testing and Materials (ASTM) 2010. Standard test method for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates, American Society for Testing and Materials. Report E1706. ASTM, Philadelphia

Auffan, M., Achouak, W., Jérôme, R., Roncato, M., Chanéac, C., Waite, D. T., Masion, A. Woicik, J. C., Wiesner, M. R. and Bottero, J 2008. Relation between the Redox State of Iron-Based Nanoparticles and Their Cytotoxicity toward *Escherichia coli*. *Environmental Science and Technology* **47**: 6730-6735

Baird, D.J., Soares, A.M.V.M., Girling, A., Barber, I., Bradley, M., Calow, P., 1989. The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. **In**: Lokke, H., Tyle, H., Bro-Rasmussen, F. (Eds.), *Proceedings of the First European Conference on Ecotoxicology*. Copenhagen, Denmark, 144–148 pp.

Barnes, D. K. A., Galgani, F., Thompson, R. C. and Barlaz, M. 2009. Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of The Royal Society B* **364**: 1985–1998.

Baun, A., Hartmann, N. B., Grieger, K. D. and Kusk, K. O. 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotoxicology* **17**: 387-395.

Besseling, E., Wang, B., Lürling, M and Koelmans, A. A. 2014. Nanoplastic Affects Growth of *S. obliquus* and Reproduction of *D. magna*. *Environmental Science and Technology* **48**: 12336–12343.

Bihari, P., Vippola, M., Schultes, S., Praetner, M., Khandoga, A. G., Reichel, C. A., Coester, C., Tuomi, T., Rehberg, M. and Krombach, F. 2008. Optimized dispersion of nanoparticles for biological in vitro and in vivo studies. *Particle and Fibre Toxicology* **5**: 207-221

Booth, A. M., Hansen, B. H., Frenzel, M., Johnsen, H. and Altin, D. 2015. Uptake and toxicity of methylmethacrylate-based nanoplastic particles in aquatic organisms. *Environmental Toxicology and Chemistry*. **2**: 45-51

Bozich, J. S., Lohse, S. E., Torelli, M. D., Murphy, C. J., Hamers, R. J. and Klaper, R. D. 2014. Surface chemistry, charge and ligand type impact the toxicity of gold nanoparticles to *Daphnia magna*. *Environmental Science: Nano* **1**: 260-270.

Bradley, M. C., Naylor, C., Calow, P., Baird, D. J. and Soares, A. M. V. M. 1993. Reducing variability in Daphnia toxicity tests – a case for further standardization. **In**: Soares, A.M.V.M., Calow, P. (Eds.), *Progress in Standardization of Aquatic Toxicity Tests*. Lewis Publishers, UK

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M. and Thompson, R. C. 2008. Ingested Microscopic Plastic Translocates to the Circulatory System of the Mussel, *Mytilus edulis* (L.). *Environmental Science and Technology* **42**: 5026–5031.

Burleson, D. J., Driessen, M. D. and Penn, R. L. 2004. On the Characterization of Environmental Nanoparticles. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering* **39**: 2707-2753.

Castañeda, R., Avlijas, S., Simard, A. and Ricciardi, A. 2014. Microplastic pollution in St. Lawrence River sediments. *Canadian Journal of Fisheries and Aquatic Sciences* **71**: 1-5.

Cauwenberghe, L., Vanreusel, A., Mees, J. and Janssen, C. R. 2013. Microplastic pollution in deep-sea sediments. *Environmental Pollution* **182**: 495–499.

Chen, X. and Schluesener, H. J. 2008. Nanosilver: A nanoproduct in medical application. *Toxicology Letters* **176**: 1–12.

Cho, E. J., Holback, H., Liu, K. C., Abouelmagd, S. A.,Park, J. and Yeo, Y. 2013. Nanoparticle characterization: State of the art, challenges, and emerging technologies. *Molecular Pharmaceutics* **10**: 2093-2110.

Christian, P., Von der Kamme, F., Baalousha, M. and Hofmann, T. 2008. Nanoparticles: structure, properties, preparation and behaviour in environmental media. *Ecotoxicology* **17**: 326-343.

Cole, M., Lindeque, P., Halsband, C. and Galloway, T. S. 2011. Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin* **62**: 2588–2597.

Daniel, M. and Astruc, D. 2004. Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. *Chemical Reviews* **104**: 293−346.

Decaestecker, E., Meester, L. D. and Mergeay, J. 2009. Cyclical Parthenogenesis in Daphnia: Sexual Versus Asexual Reproduction. **In**: *Lost Sex: The Evolutionary Biology of Parthenogenesis*. Schön, I., Martens, K. and Dijk, P., 2nd edition. Springer, Netherlands 295-316.

Dhawan, A. and Sharma, V. 2010. Toxicity assessment of nanomaterials: methods and challenges. *Analytical and Bioanalytical Chemistry* **398**: 589-605.

Doak, S., Griffiths, M., Manshian, B., Singh, N., Williams, P., Brown, P. and Jenkins, G. 2009. Confounding experimental considerations in nanogenotoxicology. *Mutagenesis* **24**: 285-293.

Dominguez, G. A., Lohse, S. E., Torelli, M. D., Murphy, C. J, Hamers, R. J., Orr, G. and Klaper, R. 2015. Effects of charge and surface ligand properties of nanoparticles on oxidative stress and gene expression within the gut of *Daphnia magna*. *Aquatic Toxicology* **162**: 1-9.

Dreaden, E. C., Alkilany, A. M., Huang, X., Murphy, C. J. and El-Sayed, M. A. 2012. The golden age: gold nanoparticles for biomedicine. *Chemical Society Reviews* **41**: 2740–2779.

Dykmana, L. and Khlebtsov, N. 2012. Gold nanoparticles in biomedical applications: recent advances and perspectives. *Chemical Society Reviews* **41**: 2256–2282.

Ebert, D. 2005. Introduction to Daphnia Biology. **In**: Ecology, Epidemiology, and Evolution of Parasitism in Daphnia. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information.

Eerkes-Medrano, D., Thompson, R. C. and Aldridge, D. C. 2015. Microplastics in freshwater systems: A review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. *Water Research* **75**: 63-82.

Elsaesser, A. and Howard, C. V. 2012. Toxicology of nanoparticles. *Advanced Drug Delivery Reviews* **64**: 129–137.

Eriksen, M., Mason, S., Wilson, S., Box, C., Zellers, A., Edwards, W., Farley, H. and Amato, S. 2013. Microplastic pollution in the surface waters of the Laurentian Great Lakes. *Marine Pollution Bulletin* **77**: 177–182.

Eustis, S. and El-Sayed, M. A. 2006. Why gold nanoparticles are more precious than pretty gold: Noble metal surface plasmon resonance and its enhancement of the radiative and nonradiative properties of nanocrystals of different shapes. *Chemical Society Reviews* **35**:

Faraday, M. 1857. The Bakerian Lecture: Experimental Relations of Gold (and Other Metals) to Light. *Philosophical Transactions of the Royal Society of London* **147**: 145-181.

Foley, S., Crowley, C., Smaihi, M., Bonfils, C., Erlanger, B. F., Seta, P. and Larroque, C. 2002. Cellular localisation of a water-soluble fullerene derivative. *Biochemical and Biophysical Research Communications* **294**: 116–119.

Fossi, M. C. and Depledge, M. H. 2014. Exploring the potential of large vertebrates as early warning sentinels of threats to marine ecosystems, human health and wellbeing. *Marine*

Environmental Research **100**: 1-2.
Free, C., Jensen, O., Mason, S., Eriksen, M., Williamson, N. and Boldgiv, B. 2014. High-levels of microplastic pollution in a large, remote, mountain lake. *Marine Pollution Bulletin* **85**: 156–163.
Freestone, I., Meeks, N., Sax, M. and Higgitt, C. 2007. The Lycurgus Cup - A Roman Nanotechnology. *Gold Bulletin* **14**: 270-277.
Frias, J. P. G. L., Sobral, P and Ferreira, A. M. 2010 Organic pollutants in microplastics from two beaches of the Portuguese coast. *Marine Pollution Bulletin* **60**: 1988-1992.
Galgani, F., Claro, F., Depledge, M. and Fossi, C. 2014. Monitoring the impact of litter in large vertebrates in the Mediterranean Sea within the European Marine Strategy Framework Directive (MSFD): Constraints, specificities and recommendations. *Marine Environmental Research* **100**: 3–9.
García-Cambero, J. P.. García, M. N., López, G. D., Herranz, A. L., Cuevas, L., Pérez-Pastrana, E., Cuadal, J. S., Castelltort, J. R. and Calvo, A. C. 2013. Converging hazard assessment of gold nanoparticles to aquatic organisms. *Chemosphere* **93**: 1194–1200.
Gargini, P. A. 2004. Silicon nanoelectronics and beyond. *Journal of Nanoparticle Research* **6**: 11–26.
Ghosh, P., Han, G., De, M., Kim, C. K. and Rotello, V. M. 2008. Gold nanoparticles in delivery applications. *Advanced Drug Delivery Reviews* **60**: 1307–1315.
Gilroy, K. D., Neretina, S. and Sanders, R. W. 2014. Behavior of gold nanoparticles in an experimental algal-zooplankton food chain. *Journal of Nanoparticle Research* **16**: 2414.
Ginzburg, V. V. and Balijepalli, S. 2007. Modeling the Thermodynamics of the Interaction of Nanoparticles with Cell Membranes. *Nano Letters* **7**: 3716-3722.
Gophen, M. and Geller, W. 1984. Filter mesh size and food particle uptake by Daphnia. *Oecologia* **64**: 408-412.
Gou, N., Onnis-Hayden, A. and Gu, A. Z. 2010. Mechanistic toxicity assessment of nanomaterials by whole-cell-array stress genes expression analysis. *Environmental Science and Technology* **44**: 5964-5970.
Haiss, W., Thanh, N. T. K., Aveyard, J. and Fernig, D. J. 2007. Determination of Size and Concentration of Gold Nanoparticles from UV−Vis Spectra. *Analytical Chemistry* **79**: 4215-4921.
Handy, R., von der Kamme, F., Lead, J. R., Hassellöv, M., Owen, R. and Crane, M. 2008. The ecotoxicology and chemistry of manufactured nanoparticles. *Ecotoxicology* **17**: 287-314
Hopewell, J., Dvorak, R. and Kosior, E. 2009. Plastics recycling: challenges and opportunities. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**: 2115–2126.
Huang, X. and El-Sayed, M. A. 2011. Plasmonic photo-thermal therapy (PPTT). *Alexandria Journal of Medicine* **47**: 1-9.
Imhof, H. K., Ivleva, N. P., Schmid, J., Niessner, R. and Laforsch, C. 2013. Contamination of beach sediments of a subalpine lake with microplastic particles. *Current Biology* **23**: R867–R868.
Jemec, A., Drobne, D., Tišler, T., Trebše, P., Roš, M. and Sepčić, K. 2007. The applicability of acetylcholinesterase and glutathione S-transferasein *Daphnia magna* toxicity test. *Comparative Biochemistry and Physiology, Part C* **144**: 303-309.
Jong, W. H. D., Hagens, W. I., Krystek, P., Burger, M.C., Sips, A. J. A. M. and Geertsma, R. E. 2008. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. *Biomaterials* **29**: 1912–1919.

species mediated membrane damage induced by fullerene derivatives and its possible biological implications. *Toxicology* **155**: 55–61.
Kee, D. and Ebert, D. 1996. The effect of temperature on maturation threshold body-lenght in *Daphnia magna*. *Oecologia* **108**: 627-630.
Khan, F. R., Kennaway, G. M., Croteau, M., Dybowska, A., Smith, B. D., Nogueira, A. J. A, Rainbow, P. S., Luoma, S. N. and Valsami-Jones, E. 2014. *In vivo* retention of ingested Au NPs by *Daphnia magna*: No evidence for trans-epithelial alimentary uptake.

Chemosphere **100**: 97–104.

Khlebtsov, N. and Dykman, L. 2011. Biodistribution and toxicity of engineered gold nanoparticles: a review of *in vitro* and *in vivo* studies. *Chemical Society Reviews* **40**: 1647-1671.

Kittler, S., Sengstock, C., Diendorf, J., Köller, M. and Epple, M. 2010. Toxicity of Silver Nanoparticles Increases during Storage Because of Slow Dissolution under Release of Silver Ions. *Chemistry Of Materials* **22**: 4548–4554.

Klaassen, C. D. (Eds.) 2013. Casarett and Doull's Toxicology: the basic science of poisons. 8th edition. McGraw-Hill Medical, US. 1309 pp.

Kruis, F. E., Fissan, H. and Peled, A. 1998. Synthesis on Nanoparticles in the Gas Phase for Electronic, Optical and Magnetic Applications - A Review. *Journal of Aerosol Science* **29**: 511–535.

Kumar, A., Zhang, X. and Liang, X. 2013. Gold nanoparticles: Emerging paradigm for targeted drug delivery system. *Biotechnology Advances* **31**: 593–606.

la Farré, M., Pérez, S. and Kantiani, L. 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. *Trends in Analytical Chemistry* **27**: 991–1007.

Lampert, W. 2006. Daphnia: Model herbivore, predator and prey. *Polish Journal of Ecology* **54**: 607–620.

Lasagna-Reeves, C., Gonzalez-Romero, D., Barria, M. A., Olmedo, I., Clos, A., Ramanujam, V. M. S., Urayama, A. V., Kogan, M. J. and Soto, C. 2010. Bioaccumulation and toxicity of gold nanoparticles after repeated administration in mice. *Biochemical and Biophysical Research Communications* **393**: 649–655.

Lechner, A. and Ramler, D. 2015. The discharge of certain amounts of industrial microplastic from a production plant into the River Danube is permitted by the Austrian legislation. *Environmental Pollution* **200**: 159-160.

Lee, B. and Ranville, J. F. 2012. The effect of hardness on the stability of citrate-stabilized gold nanoparticles and their uptake by *Daphnia magna*. *Journal of Hazardous Materials* **213**: 434–439.

Lee, W., Yoon, S., Shin, Y. and An, Y. 2015. Trophic transfer of gold nanoparticles from *Euglena gracilis* or *Chlamydomonas reinhardtii* to *Daphnia magna*. *Environmental Pollution* **201**: 10–16.

Letfullin, R. R., Iversen, C. B. and George, T. F. 2011. Modeling nanophotothermal therapy: kinetics of thermal ablation of healthy and cancerous cell organelles and gold nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine* **7**: 137–145.

Link, S. and El-Sayed, M. A. 1999. Size and Temperature Dependence of the Plasmon Absorption of Colloidal Gold Nanoparticles. *The Journal of Physical Chemistry B* **103**: 4212-4217.

Li, T., Albee, B., Alemayehu, M. B., Diaz, R., Ingham, L. and Kamal, S. 2010. Comparative toxicity study of Ag, Au, and Ag-Au bimetallic nanoparticles on *Daphnia magna*. *Analytical and Bioanalytical Chemistry* **398**: 689-700.

Lithner, D., 2011. Environmental and Health Hazards of Chemicals in Plastic Polymers and Products. University of Gothenburg, US (Ph.D. Thesis)

Lundqvist, M., Stigler, J., Elia, G., Lynch, I., Cedervall, T. and Dawson, K. A. 2008. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 14265–14270.

Lynch, I., Cedervall, T., Lundqvist, M., Cabaleiro-Lago, C., Linse, S. and Dawson, K. A. 2007. science challenge for the 21st century. *Advances in Colloid and Interface Science* **134**: 167–174.

Mark, U. and Solbé, J. 1998. Analysis of the ecetoc aquatic toxicity (EAT) database V — The relevance of *Daphnia magna* as a representative test species. *Chemosphere* **36**: 155–166.

Martínez-Jerónimo, F., Villaseñor, R., Rios, G. and Espinosa, F. 1994. Effect of food type and concentration on the survival, longevity, and reproduction of *Daphnia magna*.

Hydrobiologia **287**: 207-214.
Meent, D. 2007. Environmental Exposure Assessment **In**: *Risk Assessment of Chemicals: An Introduction.* Leeuwen, C. J. and Vermeire, T. G. (Eds.) 2nd edition. Springer, Netherlands.
McCormick, A., Hoellein, T. J., Mason, S. A., Schluep, J. and Kelly, J. J. 2014. Microplastic is an Abundant and Distinct Microbial Habitat in an Urban River. *Environmental Science and Technology* **48**: 11863-11871.
Molaei, M., Saievar-Iranizad, M. M., Taghavinia, E.N., Liu, B., Sun, H. D. and Sun, X. W. 2012. Near-white emitting QD-LED based on hydrophilic CdS nanocrystals. *Journal of Luminescence* **132**: 467–473.
Morganti, P. 2010. Use and potential of nanotechnology in cosmetic dermatology. *Clinical, Cosmetic and Investigational Dermatology* **3**: 5-13.
Nabiev, I., Mitchell, S., Davies, A., Williams, Y., Kelleher, D., Moore, R., Gun'ko, Y. K., Byrne, S., Rakovich, Y. P., Donegan, J. F., Sukhanova, A., Conroy, J., Cottell, D., Gaponik, N., Rogach, A. and Volkov, Y. 2007. Nonfunctionalized Nanocrystals Can Exploit a Cell's Active Transport Machinery Delivering Them to Specific Nuclear and Cytoplasmic Compartments. *Nano Letters* **7**: 3452-3461.
Nasser, F. and Lynch, I. 2015. Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on *Daphnia magna*. *Journal of Proteomics*: 30121-30124.
Navarro, E., Baun, A., Behra, R., Hartmann, N. B., Filser, Miao, J., Quigg, A., Santschi, P. H. and Sigg, L. 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology* **17**: 372-386.
Nel, A., Xia, T., Madler, L. and Li, N. 2006. Toxic Potential of Materials at the Nanolevel. *Science* **311**: 622-627.
Nikinmaa, M. 2014. *An Introduction to Aquatic Toxicology.* Vol I, 1st edition. Elsevier, UK, 252 pp.
Oberdörster, G., Oberdörster, E. and Oberdörster, J. 2005. Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles. *Environmental Health Perspectives* **113**: 823-839.
OECD, 2004. OECD Guideline for the Testing of Chemicals. Guideline 202: Daphnia sp. Acute Immobilisation Test, adopted 14 April 2004. Organisation for Economic Co-operation and Development, France.
OECD, 2012. OECD Guideline for the Testing of Chemicals. Guideline 211: *Daphnia magna* Reproduction Test, adopted 2 October 2012. Organisation for Economic Co-operation and Development, France.
OECD 2010. Series on the Safety of Manufactured Nanomaterials. Number 25: Guidance Manual for the Testing of Manufactured Nanomaterials. Organisation for Economic Co-operation and Development, France.
Ojea-Jiménez, I., López, X., Arbiol, J. and Puntes, V 2012. Citrate-Coated Gold Nanoparticles As Smart Scavengers for Mercury(II) Removal from Polluted Waters. *ACS Nano* **6**: 2253–2260.
O'Neal, D. P., Hirsch, L. R., Halas, N. J., Payne, J. D. and West, J. L. 2004. Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. *Cancer Letters* **209**: 171-176.

2014. Aggregation behaviour of gold nanoparticles in saline aqueous media *Journal of Nanoparticle Research* **16**: 2376.
Pan, J., Buffet, P., Poirier, L., Amiard-Triquet, C., Gilliland, D., Joubert, Y., Pilet, P., Guibbolini, M., de Faverney, C. R., Roméo, M., Valsami-Jones, E. and Mouneyrac, C. 2012. Size dependent bioaccumulation and ecotoxicity of gold nanoparticles in an endobenthic invertebrate: The Tellinid clam *Scrobicularia plana*. *Environmental Pollution* **168**: 37-43.
Patil, S., Sandberg, A., Heckert, E., Self, W. and Seal, S. 2007. Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. *Biomaterials* **28**:

4600–4607.

Pattani, V. P., Li, C., Desai, T. A. and Vu, T. Q. 2008. Microcontact printing of quantum dot bioconjugate arrays for localized capture and detection of biomolecules. *Biomedical Microdevices* **10**: 367-374.

Pek, S., Gao, S., Arshad, M. S. M., Leck, K. and Ying, J. Y. 2008. Porous collagen-apatite nanocomposite foams as bone regeneration scaffolds. *Biomaterials* **29**: 4300-4305

Pérez, S. and Beiras, R. 2010. The mysid *Siriella armata* as a model organism in marine ecotoxicology: comparative acute toxicity sensitivity with *Daphnia magna*. *Ecotoxicology* **19**: 196–206.

Pietroiusti, A., Campagnolo, L. and Fadeel, B. 2013. Interactions of Engineered Nanoparticles with Organs Protected by Internal Biological Barriers. *Small* **9**: 1557-1572.

Pissuwan, D., Niidome, T. and Cortie, M. B. 2011. The forthcoming applications of gold nanoparticles in drug and gene delivery systems. *Journal of Controlled Release* **149**: 65-71.

Plastics Europe 2015. An analysis of European plastics production, demand and waste data in Plastics – the Facts 2015. Plastics Europe: 30.

Powell, M. D. and Berry, A. J. 1990. Ingestion and regurgitation of living and inert materials by the estuarine copepod *Eurytemora affinis* (Poppe) and the influence of salinity. *Estuarine, Coastal and Shelf Science* **31**: 763-773.

Qiu, T. A., Bozich, J. S., Lohse, S. E., Vartanian, A. M., Jacob, L. M., Meyer, B. M., Gunsolus, I. M., Niemuth, N. J., Murphy, C. J., Haynes, C. L. and Klaper, R. D. 2015. Gene expression as an indicator of the molecular response and toxicity in the bacterium *Shewanella oneidensis* and the water flea *Daphnia magna* exposed to functionalized gold nanoparticles. *Environmental Science: Nano* **2**: 615-629.

Raj, S., Jose, S., Sumod, U. S. and Sabitha, M. 2012. Nanotechnology in cosmetics: Opportunities and challenges. *Journal of Pharmacy And Bioallied Sciences* **4**: 186–193.

Ramos, J. A. A., Barletta, M. and Costa, M. F. 2012. Ingestion of nylon threads by Gerreidae while using a tropical estuary as foraging grounds. *Aquatic Biology* **17**: 29-34.

Renault, S., Nathalie, M., Baudrimont, M. and Mornet, S. 2008. Impacts of gold nanoparticle exposure on two freshwater species: A phytoplanktonic alga (*Scenedesmus subspicatus*) and a benthic bivalve (*Corbicula fluminea*). *Gold Bulletin* **41**: 116-126.

Rosenkranz, P., Chaudhry, Q., Stone, V. and Fernandes, T. F. 2009. A comparison of nanoparticle and fine particle uptake by *Daphnia magna*. *Environmental Toxicology and Chemistry* **28**: 2142-2149.

Saha, K., Agasti, S. S., Kim, C., Li, X. and Rotello, V. M. 2012. Gold Nanoparticles in Chemical and Biological Sensing. *Chemical Reviews* **112**: 2739–2779.

Samuel, U. and Guggenbichler, J. 2004. Prevention of catheter-related infections: the potential of a new nano-silver impregnated catheter. *International Journal of Antimicrobial Agents* **23**: 75–78.

Sengstock, C., Diendorf, J., Simon, T., Eggeler, G., Epple, M. and Koller, M. 2011. Uptake and intracellular distribution of silver nanoparticles in human mesenchymal stem cells. *Acta Biomaterialia* **7**: 347–354.

Shaw, J. R., Pfrender, M. E., Eads, B. D., Klaper, R., Callaghan, A., Sibly, R. M., Colson, I., Jansen, B., Gilbert, D. and Colbourne, J. K. 2008. Daphnia as an emerging model for toxicological genomics. *Advances in Experimental Biology* **2**: 165-219.

Shipley, H. J., Engates, K. E. and Guettner, A. M. 2011. Study of iron oxide nanoparticles in soil for remediation of arsenic. *Journal of Nanoparticle Research* **13**: 2387-2397.

Singh, M., Manikandan, S. and Kumaraguru, A. K. 2011. Nanoparticles: A New Technology with Wide Applications. *Research Journal of Nanoscience and Nanotechnology* **1**: 1-11.

2014. Uptake and depuration of gold nanoparticles in *Daphnia magna*. *Ecotoxicology* **23**: 1172-1183.

Soares, A. M. V. M. 1989. Clonal Variation in Life-history Traits in *Daphnia magna* Strauss

(Crustacea: Cladocera). Implications for Ecotoxicology. University of Sheffield, UK, (Ph.D. Thesis)

Sonavane, G., Tomoda, K. and Makino, K. 2008. Biodistribution of colloidal gold nanoparticles after intravenous administration: Effect of particle size. *Colloids and Surfaces B: Biointerfaces* **66**: 274–280.

Sperling, R., Gil, R., Zhang, F., Zanella, M. and Parak, W. 2008. Biological applications of gold nanoparticles. *Chemical Society Reviews* **37**: 1896–1908.

Stein, J. R. 1973. *Handbook of Phycological Methods. Culture Methods and Growth Measurements*. Cambridge. Cambridge University Press, UK 7-24 pp.

Talsness, C. E., Andrade, A. J. M., Kuriyama, S. N., Taylor, J. A. and vom Saal, F. S. 2009. Components of plastic: experimental studies in animals and relevance for human health. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**: 2079-2096.

Technical Advisory Panel—GEF, 2012. Impacts of Marine Debris on Biodiversity: Current Status and Potential Solutions. Secretariat of the Convention on Biological Diversity, US.

Tedesco, S., Doyle, H., Blasco, J., Redmond, G. and Sheehan, D. 2010. Oxidative stress and toxicity of gold nanoparticles in *Mytilus edulis*. *Aquatic Toxicology* **100**: 178–186.

The Royal Academy of Engineering 2004. Nanoscience and nanotechnologies: opportunities and uncertainties. The Royal Academy of Engineering, UK, 116

Tratnyek, P. G. and Johnson, R. L. 2006. Nanotechnologies for environmental cleanup. *Nano Today* **1**: 44–48.

Tungittiplakorn, W., Cohen, C. and Lion, L. W. 2005. Engineered Polymeric Nanoparticles for Bioremediation of Hydrophobic Contaminants. *Environmental Science and Technology* **39**: 1354–1358.

Verma, A. and Stellacci, F. 2010. Effect of Surface Properties on Nanoparticle–Cell Interactions. *Small* **6**: 12-21.

Vianello, A., Boldrin, A., Guerriero, P., Moschino, V., Rella, R., Sturaro, A. and Rosb, L. 2013. Microplastic particles in sediments of Lagoon of Venice, Italy: First observations on occurrence,spatial patterns and identification. *Estuarine, Coastal and Shelf Science* **130**: 54-61.

Wang, F., Kozawa, D., Miyauchi, Y., Hiraoka, K., Mouri, S., Ohno, Y. and Matsuda, K. 2015. Considerably improved photovoltaic performance of carbon nanotube-based solar cells using metal oxide layers. *Nature Communications* **6**: 34-40

Wang, Z. L. 2000. Transmission Electron Microscopy of Shape-Controlled Nanocrystals and Their Assemblies. *The Journal of Physical Chemistry B* **104**: 1153–1175.

Williams, Y., Sukhanova, A., Nowostawska, M., Davies, A. M., Mitchell, S., Oleinikov, V., Gun'ko, Y., Nabiev, I., Kelleher, D. and Volkov, Y. 2009. Probing cell-type-specific intracellular nanoscale barriers using size-tuned quantum dots. *Small* **5**: 2581-2588.

Wray, A. T. and Klaine, S. J. 2015. Modeling the influence of physicochemical properties on gold nanoparticle uptake and elimination by *Daphnia magna*. *Environmental Toxicology and Chemistry* **34**: 860-872.

Wright, S. L., Rowe, D., Thompson, R. C., and Galloway, T. S. 2013. Microplastic ingestion decreases energy reserves in marine worms. *Current Biology* **23**: R1031-R1033.

Wright, S. L., Thompson, R. C. and Galloway, T. S. 2013. The physical impacts of microplastics on marine organisms: A review. *Environmental Pollution* **178**: 483–492.

Xia, T., Kovochich, M., Brant, J., Hotze, M., Sempf, J., Oberley, T., Sioutas, C., Yeh, J. I., Wiesner, M. R. and Nel, A. 2006. Comparison of the Abilities of Ambient and Manufactured Nanoparticles To Induce Cellular Toxicity According to an Oxidative Stress Paradigm. *Nano Letters* **6**: 1794–1807.

Xia, T., Kovochich, M., Liong, M., Mädler, L., Gilbert, B., Shi, H., Yeh, J. I., Zink, J. I. and Nel, A. 2008. Comparison of the Mechanism of Toxicity of Zinc Oxide and Cerium Oxide Nanoparticles Based on Dissolution and Oxidative Stress Properties. *ACS Nano*: 2121-2134.

Yacobi, N. R., Malmstadt, N., Fazlollahi, F., DeMaio, L., Marchelletta, R., Hamm-Alvarez, S. F., Borok, X., Kim, K. and Crandall, E. D. 2010. Mechanisms of alveolar epithelial

translocation of a defined population of nanoparticles. *American Journal of Respiratory Cell and Molecular Biology* **42**: 604-614.

Zar, J. H. 1999: Biostatistical Analysis. Pearson Prentice-Hall, Upper Saddle River, NJ., 944 pp.

Zbinden, G. and Flury-Roversi, M. 1981. Significance of the LD50-Test for the Toxicological Evaluation of Chemical Substances. *Archives of Toxicology* **47**: 77-99.

Zhang, F., Wang, P., Koberstein, J., Khalid, S. and Chan, S. 2004. Cerium oxidation state in ceria nanoparticles studied with X-ray photoelectron spectroscopy and absorption near edge spectroscopy. *Surface Science* **563**: 74–82.

Zhang, W. 2003. Nanoscale iron particles for environmental remediation: An overview. *Journal of Nanoparticle Research* **5**: 323-332.

Zhou, W., Lu, C., Guo, X., Chen, F., Yang, H. and Wang, X. 2010. Mussel-inspired molecularly imprinted polymer coating superparamagnetic nanoparticles for protein recognition. *Journal of Materials Chemistry* **20**: 880–883.